LINKS BETWEEN AVIAN BOTULISM OUTBREAKS IN WATERFOWL, HATCHING ASYNCHRONY, AND LIFE HISTORY TRADE-OFFS OF PREFLEDGLING FRANKLIN'S GULLS (*Larus pipixcan*)

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by

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"In addition to digging deeper into specialized researches, is it not time to tie some of them together?"

~ Aldo Leopold, 1933

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ABSTRACT

This study investigated factors associated with two mortality events: avian botulism in waterfowl and mortality associated with hatching asynchrony in prefledgling Franklin's gulls (*Larus pipixcan*). The initial focus of my research was on the spatiotemporal relationship between mortality of Franklin's gulls and the onset of botulism outbreaks in waterfowl, and the suitability of gull carcasses for proliferation and toxigenesis of *Clostridium botulinum*. From 1999 to 2001, dead hatch-year Franklin's gulls were by far the most abundant carcasses, and the only source of toxin-laden maggots found on transects prior to the occurrence of avian botulism in waterfowl. Nest density was a significant predictor of hatch-year gull carcass density. High density of toxic material from gull carcasses prior to the onset of botulism in waterfowl coincided with high densities of susceptible birds; hence, mortality of Franklin's gulls has the potential to be a major initiating factor for botulism outbreaks at Eyebrow Lake, Saskatchewan.

The causes of gull mortality were conditions or diseases associated with starvation, stress, or immunosuppression, and most mortality occurred in third-hatched chicks. To separate effects of laying order from effects of hatching asynchrony on prefledgling survival, a cross-fostering experiment was conducted to create clutches containing asynchronously hatching eggs of the same laying order, and of similar egg mass, egg volume, and female quality. Hatching order, independent of laying order, significantly affected survival to fledging, whereas laying order had no observable effect, indicating that intraclutch variation in egg quality does not predetermine the fate of prefledglings, and may be less important than

hatching asynchrony for survival of prefledgling Franklin's gulls. Relationships among hatching asynchrony, laying order, mass, corticosterone, immune function, growth, and survival at two stages of development were complex. Hatching asynchrony significantly affected early and late prefledgling survival, and was directly or indirectly associated with mass, corticosterone level, and cell-mediated immune responses at early and later stages of development. Both hatching asynchrony and mass appeared to play key roles in mediating life history trade-offs among cell-mediated immune function, growth, and survival. In contrast to cell-mediated immune responses, primary humoral immune response was not directly affected by hatching order or mass, nor was it associated with survival to fledging. Rather, it was associated with laying order, neonatal testosterone, corticosterone at 2 weeks, growth of leg length, and clutch initiation date, illustrating the importance of examining more than one branch of the immune system in studies of life history trade-offs. This study is a step toward using a multipronged and multidisciplinary approach to demonstrate interactions and trade-offs among life history traits, the physiological mechanisms that produce these relationships, and how these relationships may change depending on stage of development.

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TO MY MOTHER…

WHO ALWAYS PLACES THE NEEDS OF OTHERS AHEAD OF HER OWN

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LIST OF ABBREVIATIONS

A	size of the study area, i.e., total area of Franklin's gull colony
a	area surveyed on transects
AIC	Akaike's Information Criterion
AMCO	American coot
β	intoxication coefficient; the product of C and P_i
BS	brood size
C	contact rate between susceptible birds and botulinum toxin
CID	clutch initiation date
CL	confidence limits
CMI	cell mediated immunity
con	control
CORT	corticosterone
CV	coefficient of variation = *se*/estimate, as defined in program Distance 4.0, referring to estimates of density or abundance.
$\hat{D}$	estimate of the density of objects (e.g., nests, carcasses) in study area (gull colony)
df	degrees of freedom
$f(0)$	the value of the probability density function of perpendicular distances, evaluated at 0 distance; the value is equivalent to the proportion of detected objects observed at 0 distance.
FRGU	Franklin's gull
$g(x)$	detection function; the probability that an object will be detected at distance x; a critical assumption for line transect modelling is that $g(0) = 1$.
GPS	Global Positioning System
HO	hatching order
i	transect line number
IGR	instantaneous growth rate
IP	intraperitoneal
k	total number of transect lines
KW	Kruskall-Wallis test

L	the sum of all transect lengths in the surveyed area
l_i	length of line *I*
l_2, l_3	length (of TMT) at 2 and 3 weeks, respectively
LO	laying order
M_1	number of animals dying from any cause
M_2	number of animals dying of secondary poisoning from botulism
MWU	Mann-Whitney U test
$\hat{N}$	estimate of the total number of objects detected within the study area, A
n	total number of objects (nests or carcasses) detected in a survey
n_i	number of objects detected on line *I*
OR	odds ratio
$\hat{P}_a$	probability that an object will be detected within area *a*
$\hat{P}_{a_{carc}}$	probability that a carcass will be detected within area *a*
$\hat{P}_{a_{nest}}$	probability that a Franklin's gull nest will be detected within area *a*
P_i	proportion of contacts resulting in intoxication (by botulinum toxin) and death
P_m	probability of maggot development within carcasses
P_{tox}	probability of toxin concentration within maggots
PHA	phytohemagglutinin
R_o	reproductive rate of an infectious pathogen; for botulism outbreaks, it can be defined as M_2/M_1
ref	reference category
SE	standard error
SRBC	sheep red blood cells
t	time between measurements
tx	treatment
TMT	tarsometatarsus
$\hat{var}$	variance = se^2
w	the strip half-width; truncation distance beyond which objects are not recorded or not included in the analysis
W_1, W_2, W_3	Mass at neonatal stage, 2 weeks, and 3 weeks, respectively

1. GENERAL INTRODUCTION

Investigation of the ecology of disease in wild animals often requires a detailed understanding of the life history of the species involved. Diagnosis of the proximate causes of death is only a first step to understanding disease in wildlife. For instance, when a wild animal is diagnosed with an infectious disease, it is almost impossible to identify underlying mechanisms predisposing it to infection (e.g., via increased transmission), or disease (e.g., via stress and immunosuppression). Furthermore, starvation and emaciation in the absence or presence of other diseases are commonly diagnosed by wildlife pathologists, and it is impossible to determine what processes had led to this condition without having followed the animal during its lifetime. A life history approach to understanding infection and disease may be key to understanding individual predispositions to pathogens, predators, and mortality.

Everything in an animal's life history entails some sort of cost, whether the unit of currency is energy, nutrients, or time. There are basic physiological costs required for maintenance and survival, predictable costs associated with development and reproduction, and other costs associated with responses to less predictable events, such as predation attempts, infection, and disease. Not meeting the costs for any one of these processes may have serious fitness consequences in terms of reduced reproduction and/or survival. When resources are limited, physiological trade-offs may exist among multiple processes occurring simultaneously. Animals in good body condition may have sufficient stores to successfully perform two processes simultaneously, and a positive association between the processes might be observed. Conversely, an inverse relationship between the same two processes may be

observed in animals of moderate to poor condition, in which resources may be more severely limited. The condition-dependence of physiological trade-offs, although intuitive, has rarely been demonstrated, and, to my knowledge, has not been demonstrated in vertebrates.

1.1 Thesis objectives

Diagnosis of type C botulism as the cause of death in wild waterfowl is relatively straightforward, but determining why outbreaks of botulism occur has proven very difficult, due to the complex ecology of outbreaks and because factors required to precipitate outbreaks remain unclear (Wobeser & Bollinger 2003). During the mid-1990s, extensive mortality of Franklin's gulls was observed on several Canadian marshes preceding or coincident with botulism outbreaks in waterfowl (T. Bollinger, Canadian Cooperative Wildlife Health Centre [CCWHC], personal communication). The dead gulls were predominantly hatch-year birds that had died of starvation, infectious diseases, or predation (CCWHC, unpublished data). It was hypothesized that Franklin's gull carcasses, regardless of the cause of death, might provide substrate to initiate outbreaks of botulism on these wetlands. The initial focus of my research, presented in Chapter 3, was to investigate the spatiotemporal relationship between mortality of Franklin's gulls and the initiation of avian botulism in waterfowl at Eyebrow Lake, Saskatchewan, a wetland in which botulism is enzootic (Wobeser 1997a). My objectives were to determine (i) the density of nests within the Franklin's gull colony, (ii) the extent and pattern of mortality of Franklin's gulls and other species prior to botulism outbreaks, (iii) the suitability of prefledgling gull carcasses for production of botulinum toxin, and (iv) the effect of temperature on the development of maggots and toxin-laden maggots within prefledgling gull carcasses.

The focus of my research progressed to discovering why Franklin's gulls die in such large numbers, i.e., what are the proximate and ultimate causes of mortality in this species? Exploring the proximate causes of prefledgling gull mortality led not to the discovery of a specific infectious disease outbreak, but rather to a collection of diseases or conditions often associated with starvation, 'stress,' or immunosuppression (Appendix D). To understand why gulls appeared to be starved, stressed, or immunocompromised, a closer examination of the life history of the species was required. An important observation was that most of the mortality occurred among chicks that were last to hatch, and the role of hatching asynchrony for chick survival is explored in Chapter 4. More specifically, the main objective for this segment of my research was to differentiate the effects of hatching order from laying order on prefledgling survival. In Chapter 5, the mechanisms by which hatching asynchrony affects survival are further explored by examining the role of body condition, growth, immune function, and the stress hormone, corticosterone. The main objectives of this segment were to investigate (i) factors affecting condition, corticosterone levels, and immune function in neonatal chicks, and in chicks that survived to 2 weeks of age, (ii) factors affecting survival during the first week of age, and to fledging, and (iii) the role of hatching asynchrony in trade-offs among immune responsiveness, growth, and survival in prefledgling gulls.

Results of these studies are re-addressed in the General Discussion (Chapter 6) which emphasizes the importance of a multidisciplinary approach to investigating mortality in wildlife. The answer to the question, 'Why are juvenile Franklin's gulls dying?,' required more than diagnostic pathology; it required an understanding of the life history strategies that could predispose individuals to starvation, immunosuppression, disease, and mortality. It also required an understanding of the concept of physiological trade-offs among growth, immune

function, stress, and survival, as well as the concept that everything that an animal does to improve fitness may come at a cost. The chapter concludes with a discussion of how features of the normal reproductive behaviour or life history of one species may have impacts on other species in the shared environment, in this case, by inadvertently initiating outbreaks of avian botulism.

2. GENERAL METHODS

2.1 Study site

Eyebrow Lake (50°55' N, 106° 08' W) is a 900 ha wetland managed by Ducks Unlimited Canada in the mid-grass prairie region of south-central Saskatchewan. Created in the early 1970s by construction of impoundments and water control structures (Saigeon 1995), the lake has 3 basins (A, B, C in Figure 3.1a) in which water levels can be controlled. The study took place in basin C which has a maximum water depth of approximately 1 m at full supply level. Predominant emergent plants are bulrush (*Scirpus* spp.) and cattail (*Typha* spp.). The lake is utilized by thousands of Franklin's gulls, waterfowl, shorebirds, and other species for breeding, moulting, or staging, and is a nationally significant site for congregatory species according to the Important Bird Areas Criteria (Important Bird Areas of Canada, http://www.ibacanada.com). Botulism has occurred annually at Eyebrow Lake for at least the last two decades, with intermittent large epizootics (Wobeser 1997a).

2.2 Study species

Each year, 60,000-80,000 Franklin's gulls form a large breeding colony in the southernmost basin of Eyebrow Lake (Chapter 3). Franklin's gulls arrive each year in mid to late April, and remain until mid July to early August. They are exclusive marsh nesters, and construct floating nests anchored to emergent vegetation. The egg laying phase at Eyebrow Lake is roughly 3 weeks, beginning in early May, and modal clutch size is 3 eggs (Soos, unpublished data), as is typical of this species (Burger & Gochfeld 1994). Laying interval between first and second, and second and third eggs was on average 48.3 and 47.4 hours,

respectively, in Alberta (Guay 1968), and 24-48 hours in Minnesota (Burger 1974). In Minnesota, hatching intervals ranged from 3-6 hours between first and second chicks, and 1-2 days between second and third chicks (Burger & Gochfeld 1994). Hatchlings are semi-precocial, and capable of swimming for short periods soon after hatching. Both parents provide care for offspring, and regurgitate food on the nest for chicks which must compete with each other to eat. Chicks generally remain on or near the nest until fledging (Burger 1974) at about 32 to 35 days of age (Burger & Gochfeld 1994).

3. IDENTIFICATION OF PRIMARY SUBSTRATE IN THE INITIATION OF AVIAN BOTULISM OUTBREAKS[1]

3.1 Introduction

Type C avian botulism is an important disease of wild waterfowl throughout the world, particularly in North America. Factors that precipitate botulism outbreaks are unclear and outbreaks remain unpredictable (Wobeser & Bollinger 2003). The disease is caused by ingestion of neuroparalytic toxin produced by *Clostridium botulinum* type C, an anaerobic bacterium that requires protein-rich substrate for growth. *C. botulinum* produces toxin only when infected with a bacteriophage containing the genetic code for type C_1 toxin (Eklund *et al.* 1971). High temperatures and shallow, stagnant, saline water with low dissolved oxygen were believed to be favourable for outbreaks (Bell *et al.* 1955); however, many wetlands with these characteristics have no history of botulism, and outbreaks often occur on deep, well-oxygenated lakes, or in late winter or early spring (Rocke *et al.* 1999). Water temperature, pH, salinity, redox potential, and invertebrate biomass influenced the occurrence of outbreaks (Rocke *et al.* 1999; Rocke & Samuel 1999), but some factors were not consistently different between lakes with and without outbreaks (Rocke *et al.* 1999). Thus, additional factors may be involved in the initiation of outbreaks.

Botulism outbreaks have been described as having an 'initiation phase' during which toxin produced within unknown substrate becomes available to birds, and a 'propagation phase' during which carcasses of birds killed by botulism become substrate for production of

[1] A similar version of this chapter is in press as Soos & Wobeser (2004).

further toxin that reaches healthy birds via the carcass-maggot cycle (Ball *et al.* 1998). The occurrence of botulism is likely not limited by the availability of spores or susceptible birds, both of which are abundant on wetlands (Smith 1982; Williamson *et al.* 1999), but by the availability of suitable protein-rich substrate for bacterial growth and toxin production (Ball *et al.* 1998). Vertebrate carcasses are optimal substrate for *C. botulinum* toxigenesis, and for the development of blowfly larvae that provide a vehicle for transfer of botulinum toxin when ingested by susceptible birds (Bell *et al.* 1955).

The primary sources of substrate that initiate outbreaks remain unknown although hundreds of botulism outbreaks have been documented in North America since the early 1900s. Sudden availability of carcasses as a result of hailstorms (Ball *et al.* 1998), blue-green algal blooms (Murphy *et al.* 2000), or fish kills (Ball *et al.* 1998) might provide substrate to initiate some botulism outbreaks. However, many wetlands experience botulism annually, suggesting that a predictable source of initial substrate exists on these wetlands. Identification of an association between substrate availability (or abundance) and the onset of botulism would allow prediction of outbreaks on such wetlands, and development of methods for surveillance and prevention.

During the mid-1990s, extensive mortality of Franklin's gulls was observed on several Canadian marshes preceding or coincident with botulism outbreaks in waterfowl (T. Bollinger, CCWHC, personal communication). The majority of gulls were hatch-year birds that had died of starvation, bacterial or parasitic infections, or predation (CCWHC, unpublished data). It was hypothesized that gull carcasses may provide substrate to initiate outbreaks of botulism on these wetlands. The focus of this study was to determine the role of Franklin's gull mortality in the initiation of avian botulism at Eyebrow Lake, Saskatchewan, a wetland in which botulism is considered enzootic (Wobeser 1997a; Wobeser 1997b). My first objective was to

investigate the spatiotemporal relationship between gull mortality, and mortality caused by botulism; hence, I examined the density of nests within the Franklin's gull colony, and the extent and pattern of mortality of gulls and other species prior to botulism outbreaks from 1999 to 2001. My second objective was to investigate the suitability of hatch-year gull carcasses for production of botulinum toxin, and the role of temperature in the development of maggots and toxin-laden maggots within hatch-year gull carcasses.

3.2 Methods

3.2.1. Transects

Line transect methods (Gates 1979; Burnham *et al.* 1980) were used to estimate density of Franklin's gull nests and density of vertebrate carcasses. From 1999 to 2001, parallel transects were placed 100 m apart using colour-coded wooden stakes to demarcate each line (Figure 3.1). Transects were established before gulls chose nest sites each year, but were placed in areas where they had nested in the past. Total length of transects (L) for 1999, 2000, and 2001 was 18.7, 6.9, and 3.8 km, respectively, as measured with Arcview 3.1 (Environmental Systems Research Institute, New York) from coordinates obtained along each transect, using Global Positioning System (GPS) receivers (eTrex Venture, eTrex, or Garmin 38, Opathe, KS). A canoe or kayak was used to search transects during annual nest surveys and weekly mortality surveys.

3.2.2. Estimation of density and abundance of Franklin's gull nests

Surveys to count gull nests were performed after nest building was complete each year, on 19-27 May, 1999, 23-30 May, 2000, and 31 May, 2001. Nests within 5 m on both sides of each transect line were counted, and excluded if more than half their diameter extended beyond 5 m. Hence, the strip half-width, denoted as w, was 5 m for nest density estimation, giving rise to a total strip width ($2w$) of 10 m. Perpendicular distance to each nest from the

centreline was recorded in 2001. In each year, area of the gull colony was estimated in Arcview 3.1 using GPS coordinates of the colony perimeter. Nest density was estimated using the strip transect method (Burnham & Anderson 1984), a modification of the line transect method which assumes that all objects are detected within the strip width (see Appendix A, Equations A1-A3). This assumption was initially believed to be appropriate because of the narrow strip half-width of 5 m and the large size of nests (1-1.5 m in diameter above water surface). To test this assumption, the line transect method, which accounts for reduced detectability with increasing distances from the centreline (Burnham *et al.* 1980), was employed in 2001 to estimate the probability of nest detection ($\hat{P}_{a_{nest}}$) and nest density (see below). $\hat{P}_{a_{nest}}$ obtained for 2001 was used subsequently to adjust estimates of nest density and abundance for 1999 and 2000, using methods described by Buckland *et al.* (2001:37, 76; or see Appendix A, Equation A4). It was assumed that there was little variation in $\hat{P}_{a_{nest}}$ among years because nests were counted by the same primary observers during the same time of year, and under similar weather conditions and vegetation density. Variance was estimated using the Delta method, standard error was obtained with the square root of variance, and 95% confidence limits were calculated using $z_{\alpha} = 1.96$ (see Buckland *et al.* 2001:76-79, or Appendix A, Equations A5-A9).

During the nest survey in 1999, GPS coordinates of nests along transects were recorded. Arcview maps of these nest coordinates were used to divide the study area into discrete sections, referred to as 'sub-areas,' based on a qualitative assessment of nest density, i.e., zero, light, medium, or high nest density (Figure 3.1b). Each transect line was divided into discrete segments based on whether segments were within or outside (i.e., zero nest density) the gull colony. To quantitatively assess this subjective method of classification, nest

density (with measures of precision) for each sub-area was estimated using the strip transect method, and adjusted with $\hat{P}_{a_{nest}}$ for 2001 as described above. Area and *L* (the sum of all transect segment lengths) for each sub-area were employed in these estimations. These sub-areas were subsequently employed to examine the relationship between nest density and carcass density (see below).

3.2.3. Estimation of carcass density and abundance

Transects were searched for carcasses weekly from 23 May – 25 July, 1999, 21 May – 23 July, 2000, and 20 May – 22 July, 2001. Searches were concentrated within, but not restricted to, 5 m of the centreline. For each dead or sick animal found, species, date, GPS coordinates, stage of carcass decomposition, and perpendicular distance from centreline (rounded to the nearest metre) were recorded. Stage of carcass decomposition was described as fresh (i.e., recently dead), sodden (i.e., intact but rotten carcass, usually wet), early maggot development (i.e., early larval fly stages that have not penetrated the carcass), or profuse maggot development (i.e., large numbers of plump larval stages that have penetrated the carcass abdomen, occupying >50% of the abdominal cavity). For species with a sufficiently large sample size ($n > 60$), probability of carcass detection ($\hat{P}_{a_{carc}}$) and carcass density were estimated using line transect methods as described below. Although providing conservative estimates, the strip transect method (which assumes that $\hat{P}_{a_{carc}} = 1$) was used to compare carcass density estimates among all species (including those with smaller sample sizes) and to examine weekly trends in mortality. All estimates were calculated using program Distance 4.0, release 2 (Thomas *et al.* 2003) as described below.

3.2.4. Line transect methods using Distance 4.0

To estimate Franklin's gull nest density in 2001 and carcass density in 1999 to 2001,

Distance 4.0 was used to model the detection function (Burnham *et al.* 1980). Data sets for nest density estimation in 2001 and carcass density estimation for 1999 to 2001 were treated similarly. In 2001, nest density was estimated using data obtained from a single visit to transects on May 31, whereas data from weekly visits to transects were pooled to estimate overall carcass density each year. Distance data were truncated to remove outliers where relevant (carcass data only) and grouped to reduce effects of heaping (rounding errors) or violation of the assumption of complete censusing of the centreline (Buckland *et al.* 2001:17, 34-35, 103). The shape of the detection curve was estimated by fitting grouped distance data to models recommended by Buckland *et al.* (2001:45-48). The best-fitting model was chosen to estimate nest or carcass density and abundance primarily using Akaike's Information Criterion (AIC). χ^2 goodness of fit was employed to assess the adequacy of the fitted models. To improve measures of precision and estimate weekly carcass density, models for each year were modified to include post-stratification analysis by week. Density estimates for each stratum (week) were obtained using the global detection function and $\hat{P}_{a_{carc}}$ of pooled strata, while weekly confidence intervals were a function of the variation of carcass density among transects within each stratum. To calculate total carcass density up to the first detected cases of botulism in waterfowl, $\hat{P}_{a_{carc}}$ and standard error of $\hat{P}_{a_{carc}}$ obtained for the entire season of each year were employed as multipliers in a model using a uniform key function with zero adjustment terms (Buckland *et al.* 2001; Thomas *et al.* 2003). Methods to calculate total and weekly carcass density for each species using the strip transect method in Distance 4.0 were similar to those described above, except that a uniform key function with zero adjustment terms and no multipliers (thereby setting $\hat{P}_{a_{carc}}$ to 1) was employed.

For 1999 data only, gull carcass density was estimated for each sub-area categorized

by nest density. Procedures were similar to those described for nest density, except values were adjusted using $\hat{P}_{a_{carc}}$ obtained for 1999. For each transect segment within the colony, an index of gull mortality rate was calculated by dividing the detected number of sick and dead hatch-year gulls by the product of hatch rate and number of nests counted (i.e., estimated number of hatched chicks). A hatch rate of 2.48 chicks per nest (Soos, unpublished data) was used in the calculations.

3.2.5. Sampling from carcasses and sick birds on transects

Samples of maggots were collected from all profusely maggot-laden carcasses found on transects, and were frozen at -20°C until processed for detection of botulinum toxin. Carcasses with early maggot development were not tested because toxin is unlikely to be found in earlier larval stages which have not penetrated the carcass abdomen. To promptly detect the onset of botulism on Eyebrow Lake, all sick waterfowl or other birds detected anywhere within Basin C were tested for botulism. Prior to euthanasia by cervical dislocation, blood samples were collected by jugular venipuncture, and placed into CaEDTA or lithium heparin tubes. Tubes were centrifuged, and serum or plasma samples were harvested and frozen at -20°C or -75°C either immediately or following temporary storage in liquid nitrogen (-196°C), until tested for botulinum toxin. With the exception of carcasses referred to in the following section, all euthanized birds and fresh carcasses found on transects were weighed and processed for necropsy and histopathology as part of a related study (Appendix D for Franklin's gulls). Carcasses that were not fresh were not collected for necropsy, but, if still relatively buoyant, were marked with bright paint or removed to avoid recounting them the following week.

3.2.6. Fate of Franklin's gull carcasses

From late May to late July each year, recently dead hatch-year gull carcasses collected from transect surveys (1999 and 2000), and apparently healthy hatch-year gulls captured and euthanized by cervical dislocation (2001) were placed within the marsh in an area adjacent to the nesting colony. Each carcass was tethered to a flagged bamboo pole with a 1-1.5 m length of cotton thread (breaking strength <1 kg). In 2001, carcasses were weighed prior to being placed in the marsh. Disappearance of carcass, state of carcass decomposition, time to profuse maggot development, and time to sinking were recorded every 1-4 days in 1999 and 2000, and daily in 2001. Maggot samples were collected and frozen at -20°C until tested for the presence of botulinum toxin.

3.2.7. Identification of botulinum toxin

For each maggot sample, 1 g was ground with 9 ml of a penicillin-streptomycin solution (10,000 IU/ml penicillin, 10,000 µl/ml streptomycin). Following centrifugation at 4000-5000 rpm for 10 min, the supernatant was frozen until tested for toxin. Adult male CD1 mice (20-30 g), housed individually or in pairs in 15 x 25 cm cages containing corn cob litter (Bed-O'Cobs, The Andersons, Maumee, OH), were provided with food (Purina Rodent Chow 3000, Ralston Purina, St. Louis, MO) and water *ad libitum*. In 1999, two mice (test and control) were employed for each maggot or serum/plasma sample tested as described by Quortrup and Sudheimer (1943). The control mouse was injected intraperitoneally (IP) with 0.1 ml of type C botulinum antitoxin (National Wildlife Health Center, Madison, WI) 30-60 min prior to injecting both mice with either 0.1 ml of maggot solution or 0.5-1 ml of serum or plasma IP. Following injection, mice were monitored for 4-5 days. A sample was considered to be positive if the test mouse died or had signs of paresis or respiratory distress while the corresponding control did not (Quortrup & Sudheimer 1943). For samples obtained in 2000

and 2001, a single mouse was used initially for each sample; control mice were employed only for those samples in which the first mouse tested positive. Mice with respiratory distress were euthanized immediately with halothane in an enclosed chamber. All mice were euthanized at the end of the trial.

3.2.8. Water temperature

Water temperature in basin C was recorded hourly with thermistors (Optic StowAway Temp loggers, Onset, Bourne, MA) submerged within 10-20 cm of the water surface in densely vegetated areas within the marsh.

3.2.9. Statistical Analyses

The *z*-test was employed for comparisons between two density estimates (e.g., comparing estimates of carcass density within and outside the colony). The generalized χ^2 statistic (Sauer & Williams 1989) was employed using program CONTRAST (Sauer & Hines 1989) to compare estimates of nest density or carcass density among nest density categories assigned in 1999. For 1999 only, a multilevel model was used to evaluate the relationship between nest density and hatch-year gull carcass density using MLwiN version 1.1 (Multilevel Models Project, Istitute of Education, Rasbash *et al*. 2000). For this analysis, uncorrected nest and carcass density estimates for each transect segment were employed, and segments with zero nesting on the same transect line were combined. Transect was employed as a random effect in the model to account for the possibility that segments from the same transect were not independent of each other. The value for R^2 was calculated as described by Snijders and Bosker (1999) for multilevel models. A Kruskall-Wallis test was performed to compare mortality rate indices of transect segments among low, medium, and high nest density categories (Norušis 2002).

Logistic regression was employed with a random intercept model using trial and year

as random effects to determine the effect of temperature on the likelihood of gull carcasses developing maggots containing botulinum toxin (MLwiN, version 1.1). Analyses were repeated to examine the effects of water temperature on probability of maggot development alone, and on probability of toxin within maggot samples. For these analyses, the mean of daily mean temperatures of the first 7 days of each trial was employed as a dichotomized categorical variable using 20°C as a cut-off value (≥20°C = '1' and <20°C = '0'). The effects of water temperature (mean of daily mean temperatures of first 7 days of trials) on number of days to maggot development and number of days to sinking were analyzed using a mixed general linear model (PROC MIXED; SAS Institute, Cary, NC; SAS Institute 2001). Model specifications included a random effect for year and trial nested within year. To compare the occurrence of toxin within maggot samples from gull carcasses collected on transects prior to and following the onset of botulism, logistic regression was employed with a random intercept model using year as a random effect (MLwiN, Rasbash *et al.* 2000). Results were considered statistically significant at $P \leq 0.05$.

3.3 Results

3.3.1. Density and abundance of Franklin's gull nests

The area of the gull colony was 172, 141, and 156 ha in 1999, 2000, and 2001, respectively (Figure 3.1). In 2000 and 2001, transects were located exclusively within the colony, whereas in 1999, 9.3 km of the 12 transects (and 8.7 km of the 11 transects surveyed for carcasses) overlapped the colony (Figure 3.1). The number of nests counted on transects was 1091, 1508, and 637, and the estimated nest density on the colony using the strip transect method (with w = 5 m) was 117, 243, and 170 nests/ha in the 3 years, respectively. For 2001, the detection function estimated with a uniform + cosine model provided the best fit for

grouped distance data, based on the lowest AIC and highest goodness of fit P (χ^2 = 0.28, P = 0.6). See Appendix B, Figure B1 for detailed explanation of the model. According to this model, $\hat{P}_{a_{nest}}$ in 2001 was 0.88 (95% CL = 0.80-0.98); hence, on average, 12% of nests were not detected when counting nests within the 10 m strip width. Although adjusted nest density and abundance appeared higher in 2000 and lower in 1999, confidence intervals overlapped (Table 3.1).

There was a significant difference in nest density among sub-areas subjectively classified as zero (0 nests/ha, SE = 0), low (18 nests/ha, SE = 10), medium (68 nests/ha, SE = 25), and high nest density (238 nests/ha, SE = 107; χ^2 = 15.6, df = 3, P = 0.0014, Figure 3.1), supporting the use of these nest density categories in further analyses.

3.3.2. Species composition of carcasses on transects

A botulism outbreak occurred in waterfowl beginning within the first week of July in each year of the study. Franklin's gulls comprised 85-93% of the carcasses observed on the gull colony prior to the recognition of botulism outbreaks (Table 3.2). Most of the gull carcasses were hatch-year birds (97.3%, 93.3%, and 80% in 1999, 2000, and 2001, respectively). Few American coots (*Fulica americana*) and eared grebes (*Podiceps nigricollis*) were found dead prior to botulism outbreaks (Table 3.2). Within the nesting colony, carcasses containing maggots were predominantly Franklin's gulls (61/69, 88%, Table 3.2), 93% (57/61) of which were hatch-year. Carcasses found outside the colony in 1999 were predominantly eared grebes (Table 3.2), almost all of which were hatch-year (11/12); no maggot-laden hatch-year grebes were detected. Only 5 maggot-laden carcasses were found outside the colony prior to the botulism outbreak in 1999 (Table 3.2).

3.3.3. Carcass density

Modelling of the distance data for species other than Franklin's gulls was not performed because of the small number of carcasses found (Table 3.2). To compare total and weekly carcass density among species, unadjusted estimates of density (using the strip transect method with w = 10 m) were employed. The total number of carcasses found per ha (including all species) prior to the botulism outbreak in 1999 was markedly higher within (166 carcasses on 8.7 km = 9.5/ha) than outside (19 carcasses on 8.1 km = 1.2/ha) the gull colony, primarily a result of higher carcass density of hatch-year gulls within compared to outside the colony (Table 3.3, $z = 5.45$, $P < 0.001$). The density of American coot and eared grebe carcasses did not differ within versus outside the colony (Table 3.3, $z_{amco} = -0.25$, $P = 0.80$; $z_{eagr} = -0.17$; $P = 0.87$). Each year, carcass density of hatch-year gulls within the colony was significantly higher than carcass density of American coots (1999: $z = 5.29$, $P < 0.001$; 2000: $z = 7.06$, $P < 0.001$; 2001: $z = 3.56$, $P = 0.0004$) and eared grebes (1999: $z = 5.01$, $P < 0.001$; 2000: $z = 7.22$, $P < 0.001$; 2001: $z = 3.66$, $P < 0.001$). These comparisons were based on the assumption that probability of carcass detection within the 10 m strip half-width was identical among species. The observed magnitude of difference between the density of Franklin's gull carcasses and that of other species likely would be affected minimally by incorporating $\hat{P}_{a_{carc}}$ for each species in these calculations.

3.3.4. Temporal pattern of hatch-year Franklin's gull mortality

The pattern of hatch-year gull mortality on the colony was similar in each year (Figure 3.2). Mortality of hatch-year birds began with onset of hatching, and peaked in mid-June. In 1999, there appeared to be a second peak in mortality in late June (Figure 3.2a), which occurred primarily in recently fledged gulls (Soos, unpublished data). Carcass density during

peak mortality appeared higher in 2000 than in 1999 and 2001 (Figure 3.2, and see below). Each year, the apparent decline in mortality of hatch-year gulls after late June coincided with fledging, and with a rapid decline in the number of gulls present as they began southward migration. Botulism among waterfowl was first detected within 1-2 weeks after peak mortality of hatch-year gulls each year (7, 4, and 3 July in 1999, 2000, and 2001, respectively, Figure 3.2). See Appendix C for maps of weekly mortality for each year.

3.3.5. Estimation of hatch-year Franklin's gull carcass density using Distance 4.0

Sample sizes of sick and dead hatch-year gulls were sufficient to model the detection function and estimate $\hat{P}_{a_{carc}}$ and carcass density for each year (n = 210, 222, and 69 within a 20 m strip half-width in 1999, 2000, and 2001, respectively). For each year, the best model to estimate the detection function for grouped distance data was the hazard rate key function + cosine adjustment term (1999: $\chi^2 = 0.0013$, $P = 0.97$; 2000: $\chi^2 = 0.0008$, $P = 0.98$; and 2001: $\chi^2 = 0.0053$, $P = 0.94$). See Appendix B, Figure B2, for a detailed explanation of selected models. Based on these models, on average 54-66% of hatch-year gull carcasses were undetected within a 10 m strip half-width on transects each year (Table 3.4). Estimated overall carcass density of hatch-year gulls on the colony for the entire prefledgling period (early June to late July) was similar in each year (Table 3.4, $\chi^2 = 1.231$, $P = 0.54$). There also was no difference in total carcass density of hatch-year gulls up to the onset of botulism outbreaks in waterfowl (Table 3.4, $\chi^2 = 4.29$, $P = 0.12$). Estimated peak density of gull carcasses 1-2 weeks prior to the recognition of botulism in waterfowl (Figure 3.2) was nearly significantly different among the three years (Table 3.4, $\chi^2 = 5.84$, $P = 0.054$), primarily because density in 2000 was higher than that observed in either 1999 ($\chi^2 = 5.03$, $P = 0.025$) or 2001 ($\chi^2 = 4.29$, $P = 0.038$).

In 1999, estimates of the total density of hatch-year gull carcasses prior to botulism outbreaks were significantly different among sub-areas classified as zero (0.2 carcasses/ha, SE = 13.8), low (3.5 carcasses/ha, SE = 2.9), medium (9.7 carcasses/ha, SE = 5.4), and high nest density (43.9 carcasses/ha, SE = 9.0; $\chi^2 = 18.52$, df = 3, $P = 0.003$). Nest density was a significant predictor of hatch-year gull carcass density ($\chi^2 = 43.69$, df = 1, $P < 0.001$, $R^2 = 0.61$), and transect line had no significant clustering effect on transect segments ($\chi^2 = 0.014$, df = 1, $P = 0.906$). Mortality rate index was not significantly different among nest density categories (Kruskall-Wallis $\chi^2 = 5.15$, df = 2, $P = 0.076$).

3.3.6. Maggot samples from transects

Of the maggot samples collected from hatch-year gull carcasses prior to botulism outbreaks in waterfowl, 0/12, 2/6, and 2/8 were positive for botulinum toxin in 1999, 2000, 2001, respectively, whereas 3/4, 5/6, and 13/17 of samples contained toxin after the onset of botulism in each year, respectively. Analysis of pooled data employing year as a random effect revealed that maggots collected after the final week of June were 19.3 times more likely to contain toxin than maggots collected prior to that time period (95% CL = 4.8-78.1, $\chi^2 = 17.17$, df = 1, $P < 0.001$). In 2000 and 2001, toxin was present in gull carcasses at least 10-11 days prior to the first detected cases of botulism in waterfowl. None of the maggot samples collected from other species prior to botulism outbreaks tested positive for toxin.[2]

3.3.7. Fate of Franklin's gull carcasses

Only two of 120 hatch-year gull carcasses placed in the marsh were removed by a scavenger (northern harrier, *Circus cyaneus*), and one carcass disappeared following a windy

[2] Total numbers of carcasses with maggot development for each species are displayed in parentheses in Table 3.2. Only carcasses with profuse maggot development were tested for toxin. Hence, samples tested in species other than FRGU were as follows: 1999: 0/1 American coot within colony, 0/3 off colony; 0/1 eared grebe off colony; 2000: 0/1 black-crowned night heron; 2001: 0/1 muskrat.

day (Table 3.5). All other carcasses remained in position and sank on average 9.1 days after placement (SD = 3.4, range = 2-20 days). The mean time required for profuse maggot development was 4.6 days (SD = 1.7, range = 1-8 days). The time to maggot development and to carcass sinking both decreased with increasing water temperature (b = -0.60 day/°C, 95% CL = -0.83 to -0.37, df = 54, $P < 0.001$, and b = -0.95 day/°C, 95% CL = -1.35 to -0.55, df = 103, $P < 0.001$, respectively).

Overall, 56.0%, 23.3%, and 67.5% of carcasses became maggot-laden in 1999, 2000, and 2001, respectively, and 12.5%, 60.0%, and 74.1% of maggot samples tested were positive for botulinum toxin each year (Table 3.5). Carcasses were 22.7 times more likely to develop toxin-laden maggots when mean daily water temperatures were ≥20°C than when temperatures were <20°C (95% CL = 3.0-170.4; $\chi^2 = 9.24$; $P = 0.002$). This was primarily because carcasses were 22.2 times more likely to become maggot-laden when water temperatures were ≥20°C (95% CL = 2.4-209.2; $\chi^2 = 8.24$; $P = 0.007$). Of the maggot-laden carcasses, there was no significant difference in the occurrence of toxin within maggots developing below or above 20°C ($\chi^2 = 0.081$; $P = 0.78$). In each year, the proportion of carcasses developing maggots or toxic maggots appeared to increase with trial, likely because of increasing temperature and increasing mass of hatch-year gulls as the season progressed. The hatch-year gulls used in trials in 2001 were representative of those found on transects as they exhibited similar trends in mean mass over time (Figure 3.3). The timing of toxin availability in carcasses was also similar to that observed on transects. In 2000 and 2001, toxin was first detected in hatch-year gull carcasses from trials initiated 11-15 days prior to the onset of botulism in waterfowl (Table 3.5). No maggot samples prior to the detection of botulism in ducks were available for testing from trials performed in 1999 (Table 3.5).

3.4 Discussion

The initial source of substrate for proliferation and toxigenesis of *C. botulinum* in the initiation phase of avian botulism outbreaks is typically unknown. A botulism outbreak occurred among waterfowl at Eyebrow Lake, Saskatchewan, beginning in the first week of July each year of the study. Dead hatch-year gulls were by far the most abundant vertebrate carcasses found prior to each outbreak. The pattern of hatch-year gull mortality was similar each year, with mortality peaking 1-2 weeks prior to the first detected case of botulism in waterfowl. Hatch-year gull carcasses were suitable substrate for production of toxin by *C. botulinum* type C, and were the predominant source of substrate for both toxin production and maggot development on transects, beginning at least 10-11 days prior to the first detected cases of botulism in waterfowl.

The density or abundance of carcass substrate during the initiation phase of avian botulism outbreaks is likely an important factor determining whether an outbreak will progress to the propagation phase. High carcass density may perpetuate the carcass-maggot cycle by overwhelming the scavenging system, and increasing the contact between toxin laden maggots and susceptible birds (Wobeser 1997a). The estimated density of hatch-year gull carcasses during the peak of mortality ranged from 8.4 to 18.7 carcasses/ha (Table 3.4), and scavenging did not play an important role in reducing carcass density in the marsh (Table 3.5). Enclosures with 12.5 duck carcasses/ha were on average 4.5 times more likely to develop botulism outbreaks than enclosures with no carcasses (Reed & Rocke 1992), and radiotracked mallard ducks were 12.5 times more likely to die within areas containing 12 carcasses/ha than within areas with 0 carcasses/ha on lakes high at risk for botulism outbreaks (Evelsizer 2003).

The probability of maggot development within carcasses (P_m) and the probability of toxin concentration within maggots (P_{tox}) may also be important factors affecting the

occurrence of a botulism outbreak (Wobeser 1997a). The reproductive rate of a botulism outbreak (R_o) is the average number of birds dying of secondary poisoning (M_2) divided by the number of animals dying for any reason (M_1), and $M_2 = M_1(P_m)(P_{tox})(\beta)$ (modified from Wobeser 1997a); hence, $R_o = \beta(P_m)(P_{tox})$. The intoxication coefficient, β, is the product of contact rate between susceptible birds and toxic material, C, and proportion of contacts resulting in intoxication and death, P_i (Wobeser 1997a). If R_o is > 1, then mortality due to botulism will be amplified. For example, during the week prior to the first detected cases of botulism in 2001, hatch-year gull carcasses had a 0.9 (9/10) probability (P_m) of developing maggots which had a 0.67 (6/9) probability (P_{tox}) of containing toxin (See Table 3.5, June 30, 2001). R_o for hatch-year gull carcasses was therefore (0.9)(0.67)(β) or 0.6β; hence, for R_o to be >1, β was >1.7. If each contact resulted in death, >1.7 contacts per hatch-year gull carcass (on average) would have been required to initiate a botulism outbreak in 2001. Contact rate was likely high during the week prior to botulism in 2001 because there was approximately 93 kg of toxic maggot-laden carcass material from hatch-year gulls within the colony, [3] concurrent with a high density of susceptible birds.

For a given lake and population density of susceptible birds, there may be a 'threshold' carcass density below which botulism outbreaks are unlikely to occur. We could not assess this because a botulism outbreak (preceded by high gull carcass density) occurred every year of this study. If carcass density is a key factor initiating botulism outbreaks, attempts should be made to investigate the relationship between population density and the density of toxic material (hence, taking into account P_m, P_{tox}, and carcass mass). Other factors such as aquatic

[3] estimated using the product of adjusted carcass density (4.6 carc/ha), mean carcass mass (214 g/carc), P_m (0.9), P_{tox} (0.67), and colony area (156 ha).

invertebrate abundance, temperature, redox potential, pH, and salinity should be considered; however, further research is required to evaluate their relationship with density of toxic material, or the effects of abiotic factors on P_m and P_{tox}.

Temperature is important in the ecology of botulism outbreaks because it influences *C. botulinum* type C proliferation and toxigenesis, and blowfly activity. Optimal temperatures for proliferation and toxigenesis are >30°C (Cato *et al.* 1986; Smith & Turner 1987); however, low levels of toxin production may occur at 12.5°C (Haagsma 1973). Gull carcasses were 22.2 times more likely to develop maggots when average water temperature was ≥20°C than when <20°C, whereas the occurrence of toxin within maggot samples did not differ above and below 20°C. The presence of toxin was only measured in maggots and not in carcasses, so that the effect of temperature on toxin production within carcasses that did not develop maggots was unknown. It was not known whether carcass temperature was independent of ambient temperatures during periods of high maggot activity, as occurs in duck carcasses (Wobeser & Galmut 1984). Hatch-year gull carcasses, being much smaller than duck carcasses, have a larger surface area to volume ratio, and are likely more affected by environmental temperatures. With increasing water temperature, gull carcasses became infested with maggots more rapidly (0.60 day earlier for each rise of 1°C), but were available for a shorter period of time before sinking (0.95 day less per increase of 1°C). These effects were probably due to increased blowfly activity and more rapid larval development, with more rapid consumption of carcasses at higher temperatures.

It is also possible that the presence of hatch-year gull carcasses, with associated proliferation of *C. botulinum*, increased spore density in the environment and in tissues of animals living in Eyebrow Lake (particularly in areas of high carcass density). This was

evident in the increase in P_{tox} over time observed in trials (Table 3.5) and on transects (e.g., maggots from gull carcasses were 19.3 times more likely to contain toxin when collected after the final week of June compared to samples collected earlier).

While I did not assess all possible sources of initial substrate (e.g., invertebrate carcasses or proteinaceous material), hatch-year Franklin's gull carcasses increased the risk of botulism outbreaks at Eyebrow Lake in 1999-2001, given their extensive mortality, their suitability as substrate for *C. botulinum* proliferation and toxigenesis, the substantial biomass of toxic material provided by their carcasses, and the consistent timing of mortality and availability of toxin-laden maggots prior to botulism outbreaks in waterfowl.

3.5 Management implications

The most widely used technique to manage avian botulism has been collection and disposal of carcasses during the propagation phase of outbreaks. Carcass removal is labour-intensive, costly (Ball *et al.* 1998), inefficient at reducing carcass densities (Cliplef & Wobeser 1993), and ineffective at reducing duck mortality rates due to botulism (Evelsizer 2003). Once botulism outbreaks have progressed to the propagation phase, mortality is likely to exceed the rate at which carcasses can be collected. Carcass removal might be more successful if performed during the initiation phase of outbreaks, when carcass density and mortality are lower. This requires early surveillance. Outbreaks of botulism on Eyebrow Lake from 1988 to 1992 were first detected between 14-28 July, with a median date of 21 July (Saigeon 1995); however, during each year of this study, the first cases of botulism were detected within the first week of July, and mortality had expanded into a large outbreak by mid to late July. If management is deemed necessary on lakes where botulism occurs frequently, intensive surveillance should begin prior to the anticipated onset of botulism, and should concentrate on identifying sources of substrate, and locating areas with high carcass densities, such as nesting

areas of colonial species. Such areas could be targeted for carcass removal long before the expected onset of botulism outbreaks. Management strategies also might focus on preventing birds from nesting in high densities, or on reducing hatch rate in abundant species that generate high juvenile carcass densities. Such strategies should be evaluated for their effectiveness in reducing mortality caused by botulism prior to being incorporated into management plans.

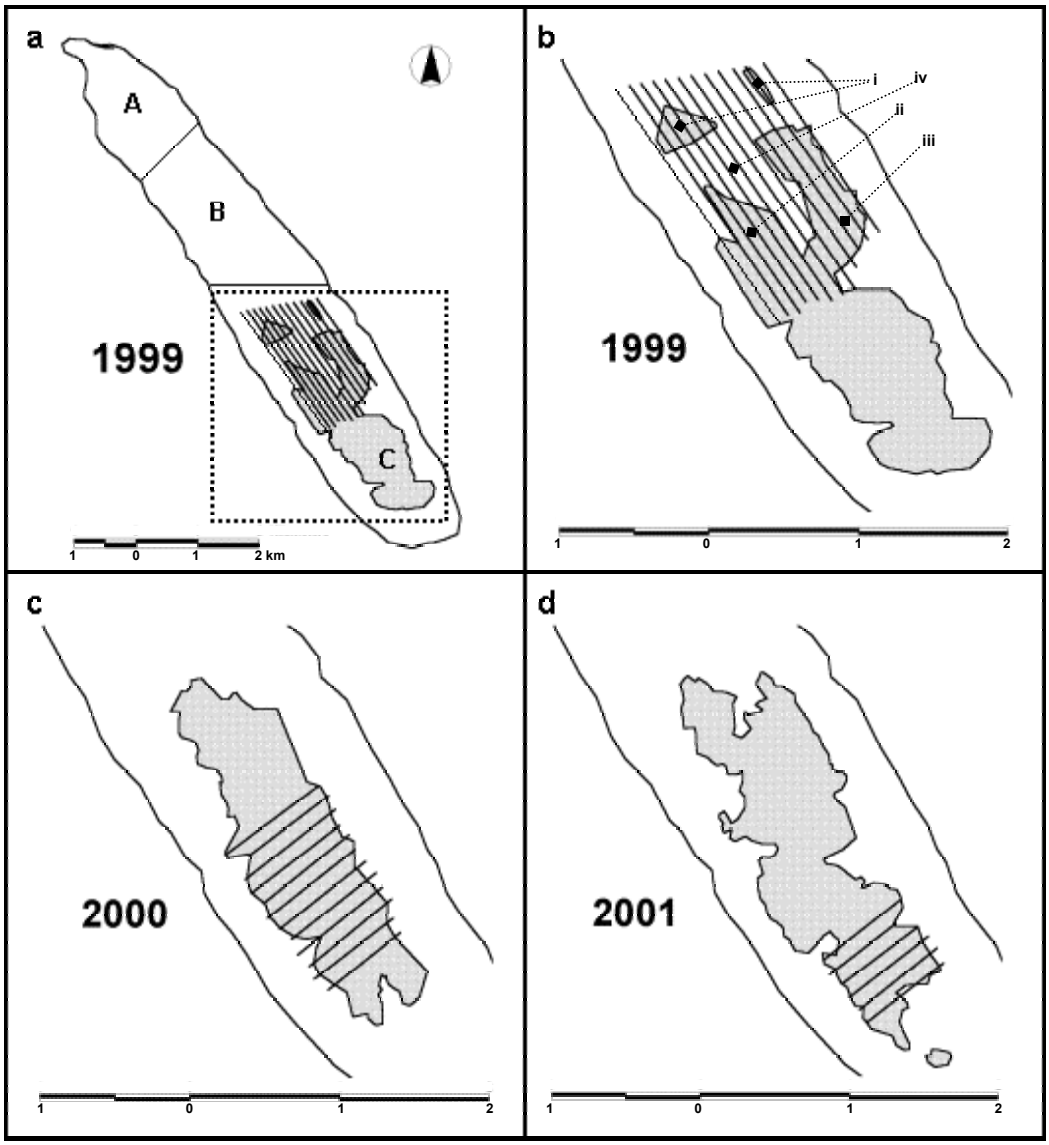

Figure 3.1. Map of Eyebrow Lake showing location of basins and Franklin's gull colony in 1999, 2000, and 2001. Solid outermost outline represents the shoreline of Eyebrow Lake, and shaded area on lake represents the area of the gull colony. Parallel lines indicate location of transects surveyed each year. Box with dashed outline in (a) represents the area on the lake shown in (b) through (d). In (b), numerals i, ii, iii, and iv represent sub-areas within the study area categorized as light, medium, high, and zero nest density, respectively. The border between sub-areas ii and iii was located between the 6th and 7th transects (from east to west). Western-most transect (1.9 km) in 1999 was not included in surveys for mortality. Northern-most transect (0.7 km) in 2000 was not included in nest survey.

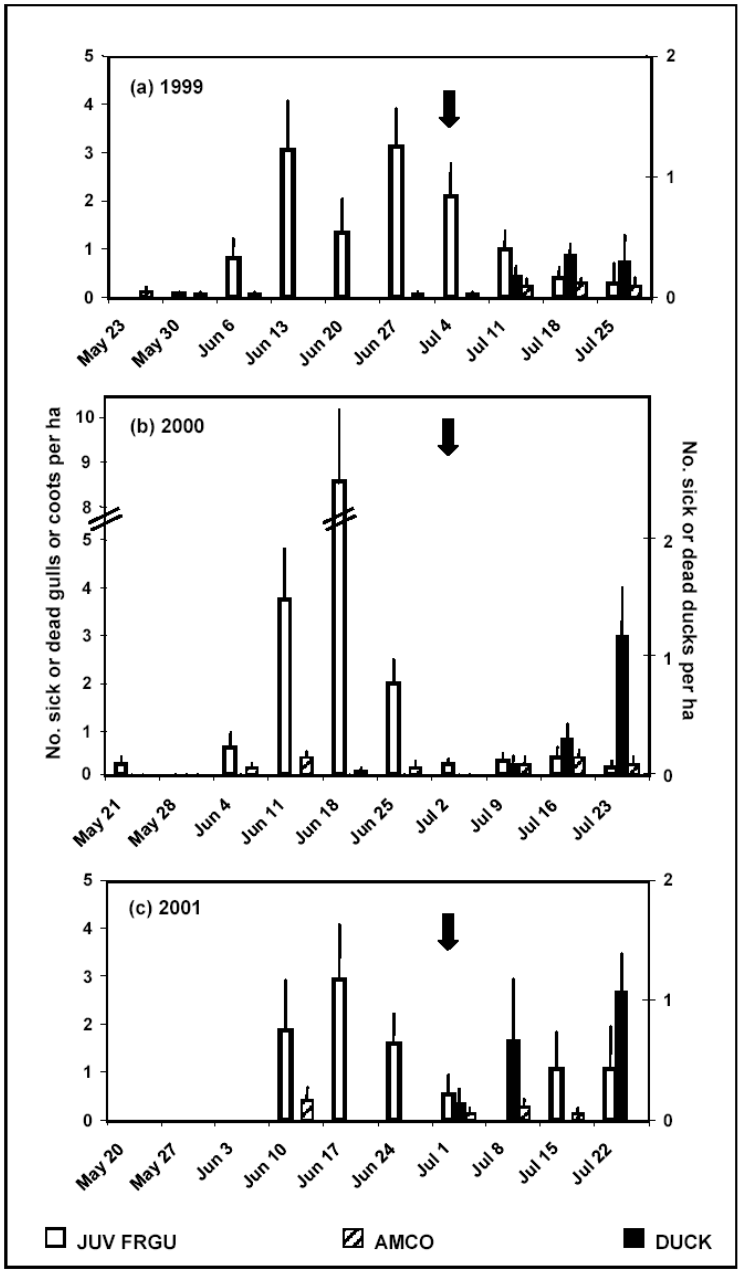

Figure 3.2. Weekly pattern of mortality of hatch-year (juv) Franklin's gulls, American coots, and dabbling ducks on the gull colony in (a) 1999, (b) 2000, and (c) 2001. Number of sick or dead birds per ha (+ 1 SE) was estimated with strip transect methods, using a 10 m strip half-width. Black arrows indicate week during which the first botulism cases in waterfowl were detected within the study area. The first case of botulism was detected along a transect line approximately 100 m outside the gull colony in 1999, within the colony but between transects in 2000, and within the colony along a transect line in 2001.

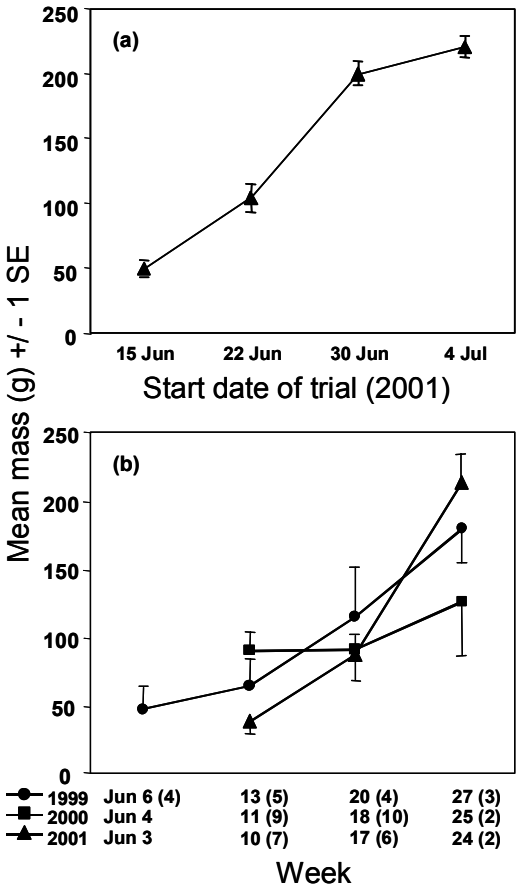

Figure 3.3. Mean mass of Franklin's gull chicks (a) employed in the fate of gull carcass study in 2001 (*n* = 10 for each trial), and (b) collected on transects in 1999-2001. Sample sizes in (b) are displayed in parentheses.

Table 3.1. Adjusted estimates of Franklin's gull nest density (nests/ha) and abundance in 1999, 2000, and 2001. Estimates for 2001 were obtained from the model created in Distance 4.0. The probability of nest detection ($\hat{P}_{a_{nest}}$) for 2001 (0.88) was employed to adjust nest density estimates for 1999 and 2000.

Year	Adjusted nest density			Adjusted no. of nests			CV
	$\hat{D}$	SE	95% CL	$\hat{N}$	SE	95% CL	
1999	133	48	61 - 290	22,789	8,204	10,433 - 49,777	0.36
2000	276	41	207 - 367	38,949	5,726	29,249 - 51,864	0.15
2001	192	22	147 - 252	30,056	3,499	22,984 - 39,302	0.12

Table 3.2. Species composition of carcasses found within a 10 m strip half-width on transects prior to botulism outbreaks each year. Number of carcasses with maggot development are indicated in parentheses for each species.

Species	Off colony	Within colony		
	1999	1999	2000	2001
Franklin's gull	1 (1)	149 (26)	223 (26)	60 (9)
American coot	6 (3)	5 (4)	10 (1)	3 (0)
Eared grebe	12 (1)	11 (1)	6 (0)	2 (0)
Other[a]	0 (0)	1(0)	1 (1)	6 (1)
Total	19 (5)	166 (31)	240 (28)	71 (10)

[a] Other species consisted of a redhead, *Aythya americana* in 1999, a black-crowned night heron, *Nycticorax nycticorax* in 2000, and a common tern, *Sterna hirundo*, a yellow-headed blackbird, *Xanthocephalus xanthocephalus*, and 4 muskrats, *Ondatra zibethicus* (of which 1 was maggot-laden) in 2001.

Table 3.3. Unadjusted estimates of total carcass density (carcasses/ha) within Franklin's gull colony on Eyebrow Lake, Saskatchewan, prior to the discovery of botulism in waterfowl. Estimates were calculated with the strip transect method, using a 10 m strip half-width. The 95% confidence limits are shown in parentheses.

Species	Off colony	Within colony		
	1999	1999	2000	2001
HY Franklin's gull	0.058	8.4	15.1	6.4
	(0.005-0.7)	(5.8-12.1)	(11.5-19.9)	(3.7-11.1)
American coot	0.35	0.29	0.73	0.40
	(0.1-1.0)	(0.1-0.7)	(0.4-1.4)	(0.08-2.0)
Eared grebe	0.69	0.63	0.44	0.27
	(0.3-1.4)	(0.3-1.4)	(0.2-0.9)	(0.06-1.1)

Table 3.4. Estimates of carcass detection probability ($\hat{P}_{a\,carc}$) and carcass density (carcasses/ha) of hatch-year Franklin's gulls within the colony on Eyebrow Lake in 1999, 2000, and 2001 using Distance 4.0. The 95% confidence limits are shown in parentheses.

Year	$\hat{P}_{a\,carc}$	Overall carcass density[a]	Total carcass density to botulism outbreak[b]	Peak carcass density[c]
1999	0.34	35.1	24.3	9.0
	(0.29-0.41)	(25.2-48.9)	(16.2-36.3)	(5.1-15.9)
2000	0.46	35.1	32.9	18.7
	(0.41-0.51)	(26.5-46.6)	(24.4-44.5)	(12.4-28.2)
2001	0.35	26.4	18.3	8.4
	(0.27-0.45)	(15.8-44.0)	(10.0-33.8)	(3.2-21.9)

[a] Includes all hatch-year Franklin's gulls detected from early June to late July.

[b] Includes all hatch-year Franklin's gulls detected from early June to the first detected case of botulism each year, i.e., the beginning of July.

[c] Estimates of peak carcass density correspond to peaks in Fig. 3.2. These estimates may be conservative because they represent newly detected carcasses in a single week, and do not include carcasses from the previous week that might have persisted had they not been removed.

Table 3.5. Fate of recently dead hatch-year Franklin's gull carcasses placed in Eyebrow Lake in 1999, 2000, and 2001. Ten carcasses were employed for each trial unless otherwise stated.

Year	Start dates	Early maggot develop-ment only	Maggot-infested	No. positive/ no. tested for toxin	No. days to maggot infestation $\bar{x}$ (range)	No. days to sinking $\bar{x}$ (range)	Water Temperature (°C) Average Daily[a] $\bar{x}$	Max
1999								
	15 Jun[b]	5	0	0/0	NA[c]	8.7 (6-10)	22.3	26.0
	29 Jun[d]	1	0	0/0	NA	9.8 (7-13)	19.5	22.5
	6 Jul	2	8	0/6	5.3 (3-6)	7.6 (6-8)	22.6	27.9
	15 Jul	0	10	0/5	4.8 (4-5)	7.0 (7)	21.8	25.9
	19 Jul	0	10	2/5	2.2 (1-4)	5.0 (4-6)	24.3	29.3
2000								
	14 Jun	0	0	0/0	NA	13.9 (8-20)	16.5	18.4
	19 Jun	1	3	1/1	8 (8)	14.5 (10-15)	17.2	19.1
	21 Jun	3	4	2/4	6.5 (3-8)	11.3 (7-13)	17.2	19.3
2001								
	15 Jun	0	0	0/0	NA	6.1 (2-7)	19.5	22.8
	22 Jun	1	9	6/9	4.7 (4-7)	7.9 (7-8)	21.3	25.8
	30 Jun[e]	0	9	6/9	4.7 (3-6)	8.7 (6-9)	20.8	28.0
	6 Jul[e]	0	9	8/9	4.0 (3-7)	8.7 (7-10)	22.8	26.7

[a] Temperatures represent average daily mean or maximum water temperatures of first 7 days of each trial.
[b] Eleven carcasses were employed in this trial; 4 were put out on 17 June. One carcass disappeared following windy day.
[c] Not applicable (i.e., carcasses did not become maggot-infested).
[d] Nine carcasses were employed in this trial.
[e] One carcass from each of the latter two trials in 2001 was removed by a northern harrier within one day of placement in the marsh.

4. HOW IMPORTANT IS LAYING ORDER RELATIVE TO HATCHING ORDER FOR CHICK SURVIVAL?[1]

4.1 Introduction

In recent years, there has been a surge of ecological studies exploring the role of maternal effects and egg quality on offspring fitness. It has been hypothesized that egg size variation and the differential allocation of yolk constituents into eggs within a clutch are adaptive, and have profound effects on offspring growth, immune function, development, or survival (Slagsvold *et al.* 1984; Schwabl 1996; Royle *et al.* 1999; Royle *et al.* 2001; Blount *et al.* 2002; Royle *et al.* 2003). Carotenoids or other yolk constituents have been shown to affect chick immune function (Haq *et al.* 1996; Fenoglio *et al.* 2002; Saino *et al.* 2003) and growth (Schwabl 1993; Schwabl 1996; Eising *et al.* 2001; Hayward & Wingfield 2004); however, the consequences of within clutch variation of egg components to offspring survival are generally unknown.[2] Indicators of egg quality, which include egg size (as measured by mass or volume), yolk mass, and concentrations of components such as albumen, lipids, cholesterol, carotenoids, and immunoglobulins, have been shown to decline with laying order in a number of species (Meathrel & Ryder 1987; Royle *et al.* 1999; Royle *et al.* 2001; Blount *et al.* 2002; Saino *et al.* 2002). The decline in egg quality observed with laying order may result in chicks of lesser quality hatching from eggs later in the laying sequence, placing them at a competitive

[1] A similar version of this chapter will be submitted for publication as Soos *et al.* (2004a).
[2] An exception is testosterone, which has been shown to decrease offspring survival in American kestrels (*Falco sparverius*, Sockman & Schwabl 2000).

disadvantage to their siblings. In species with asynchronous hatching of eggs, it is unclear whether the effects of laying order are additive to the effects of hatching asynchrony.

The decrease in egg size that occurs with laying order in many species creates a size hierarchy among nestlings, which is exacerbated by hatching asynchrony, resulting in last-hatched chicks with significantly lower weights than those of their older siblings (Pierotti & Bellrose 1986; Magrath 1992; Williams 1994; Aparicio 1999). As a result of this age and size hierarchy, last-hatched chicks have reduced competitive abilities relative to their siblings, and consequently have higher mortality rates (Pierotti & Bellrose 1986; Bollinger *et al.* 1990; Sydeman & Emslie 1992; Stoleson & Beissinger 1995). In general, hatching asynchrony is thought to be considerably more important than egg size in creating size hierarchies within broods (Magrath 1992; Bollinger 1994; Wiebe & Bortolotti 1996; Royle & Hamer 1998). It has also been hypothesized that females can 'fine-tune' the effects of hatching asynchrony, either by favouring chicks with the highest reproductive value and facilitating brood reduction, e.g., by sequentially reducing the level of carotenoids, lipids, or immunoglobulins with laying order (Royle *et al.* 1999; Royle *et al.* 2001; Blount *et al.* 2002), or by counteracting the effects of hatching asynchrony, e.g., by increasing yolk androgens with laying order, thereby improving competitive ability of last-hatched chicks (Schwabl 1993; Schwabl 1996; Eising *et al.* 2001; Royle *et al.* 2001; Groothuis & Schwabl 2002). If the size hierarchy created by hatching asynchrony is the major determinant of prefledgling survival, what is the relative importance of laying order and its effects on egg mass and other measures of egg quality to survival of hatchlings?

The main objective of this study was to differentiate the effects of hatching order from laying order on prefledgling survival of Franklin's gulls. I conducted a cross-fostering

experiment to create clutches containing asynchronously hatching eggs of the same laying order, in an attempt to create clutches of eggs similar in quality. I predicted that egg effects associated with laying order on prefledgling survival would be overridden by effects of hatching order.

4.2 Methods

4.2.1. Experimental procedures

At the beginning of the egg-laying phase from 7-17 May, 2001, 333 nests containing one egg were each marked with a flagged bamboo pole placed 2-3 m north of each nest. Nests were visited every second day until clutch completion to determine lay dates and laying order of subsequent eggs. Each egg was weighed (nearest 0.5 g), measured (maximum breadth and length, nearest 0.1 mm), and marked with indelible ink on the day it was found, and egg volume (V) was estimated using $V = 0.000476 \times L \times B^2$, where L = length and B = breadth (Bolton 1991). Only nests containing 3 eggs of known laying order (henceforth denoted as a-, b-, and c-eggs for first, second, and third-laid eggs, respectively) were included in the study, and those that appeared abandoned or disturbed were excluded. Of the remaining nests, 102 were randomly selected for detailed investigations. Of these, 72 were treatment nests employed in a cross-fostering experiment to separate the effects of hatching order from laying order. From 1-3 June, eggs were switched among treatment nests to obtain clutches containing eggs of the same laying order, i.e., 24 clutches composed of 3 a-eggs, 24 clutches of 3 b-eggs, and 24 clutches of 3 c-eggs. This was performed by assigning each of the 72 treatment nests into a group of 3 nests among which eggs were switched; nests within a group differed in clutch initiation date by 2 days, thereby ensuring asynchronous hatching of the switched eggs. Hence, biological siblings were each assigned to the same hatching order

within their respective foster nests. A 2-day interval was used to mimic the natural laying interval between eggs (Guay 1968). It was assumed that effects of clutch initiation date on egg quality of nests within a group were minimal because clutches were initiated within a 4-day period. Nests in each group were generally within 20 m of each other, in areas of similar nest and vegetation density. Thirty nests, in which eggs were similarly handled but not exchanged, were used as controls. Each of the 102 nests was enclosed within a fence to facilitate locating chicks, and to prevent chicks from moving between nests. Fences were approximately 2 m in diameter, emerging 0.4-0.6 m above the water surface, and constructed with dark green fine plastic mesh anchored with bamboo poles and short wooden stakes (Figure 4.1). Observations and manipulations were performed from a kayak.

During the hatching period (1-15 June for fenced nests), each enclosed nest was visited daily to determine hatching date and order. To identify individual chicks within a nest, the lateral surface of the right tarsometatarsus or downy feathers under the right wing were marked with coloured indelible ink; coloured plastic leg bands were fitted within 1-2 weeks of age. Chicks were handled at 0-2 days and 2 weeks of age to obtain biometric measurements and assess immune function as part of a related study (Chapter 5). Nests were inspected for mortality every 1-2 days to record mortality and fledging dates. Methods and results of gross necropsy and histopathological analyses on chicks found dead within enclosures are presented in Appendix D.

4.2.2. Statistical analyses

Since egg mass and volume were not normally distributed, Friedman's test, a nonparametric test for related samples, was employed to test for differences in these variables among siblings within all original clutches at the start of the experiment. Post-hoc

comparisons between laying order categories were performed using Dunn's test in Prism 4.01 (Motulsky 1999). Differences in egg mass and volume among hatching order groups of treatment and control clutches, and differences in total clutch mass and volume among hatching order groups for treatment clutches were similarly examined. Differences in hatching, disappearance, and fledging rates between treatment and control groups were assessed using generalized estimating equations to account for clustering by nest (PROC GENMOD, SAS Institute, Cary, N.C., SAS Institute 2001). Data were modeled with a binomial distribution and a logit link function. Median fledging age was compared among hatching order groups using the Kruskall-Wallis (KW) test.[3] Mann-Whitney U (MWU) tests were employed to compare total clutch mass, total clutch volume, hatching intervals, and hatch spread between control and treatment clutches.

Kaplan-Meier survival curves were used to illustrate the effects of hatching and laying order on survival rates over time using SPSS 11.5 (Norušis 2002), but statistical analysis was not performed on the curves. Rather, to control for clustering effect of original nest, generalized estimating equations using a logit link function were used to determine effects of hatching order, laying order, group (treatment/control), and interactions between group and hatching order on overall survival (PROC GENMOD, SAS Institute 2001). Specific contrasts between treatment and control groups for each hatching order category were also performed within the same analysis. Odds ratios were calculated using exp(b). For treatment birds only, differences in survival among a-, b-, and c-chicks were examined separately for each hatching order category using Pearson's χ^2 analysis or exact test where appropriate (PROC

[3] Repeated measures ANOVA or Friedman's test would have been more ideal to account for relatedness of individuals at the nest-level. However, these analyses require balanced data (i.e., the same number of chicks per nest), which would have significantly reduced sample size because not all chicks hatched or fledged.

LogXACT 5, Cytel Software Corporation, Cambridge MA, SAS Institute 2001).

4.3 Results

4.3.1. Egg mass and volume

Mass and volume of eggs from 102 nests examined at the start of the experiment differed significantly among siblings within clutches (mass: Friedman $\chi^2 = 71.0$, df = 2, $P < 0.0001$; volume: Friedman $\chi^2 = 61.5$, df = 2, $P < 0.0001$). B-eggs were the largest, a- and b-eggs were the heaviest, and c-eggs were the smallest and lightest eggs within clutches (difference in rank sum for egg volume: a- vs. b-egg = -47.5, $P < 0.01$; a- vs. c-egg = 64.0, $P < 0.001$; b- vs. c-egg = 111.5, $P < 0.001$; egg mass: a- vs. b-egg mass = -11.0, $P > 0.05$; a- vs. c-egg mass = 96.5, $P < 0.001$; b- vs. c-egg mass = 107.5, $P < 0.001$). Overall median mass and volume for a-, b-, and c-eggs, were 38.0 g and 33.5 mm^3, 38.0 g and 34.2 mm^3, and 36 g and 32.2 mm^3, respectively. Median total clutch mass and volume were not significantly different between treatment (mass = 111.3 g, range 98.3 – 137.0 g; volume 99.1 mm^3, range 88.2 – 119.9 mm^3, $n = 72$) and control nests (mass = 111.0 g, range 100.3 – 135.8 g, MWU test: $z = -0.668$, $P = 0.50$; volume 98.3 mm^3, range 87.4 – 115.7 mm^3, $n = 27$, MWU test: $z = -0.008$, $P = 0.99$). Following cross-fostering, there was no difference in egg mass or volume among assigned hatching order groups within foster nests (mass: Friedman $\chi^2 = 1.99$, df = 2, $P = 0.37$; volume: Friedman $\chi^2 = 1.36$, df = 2, $P = 0.51$, $n = 72$); hence, treatment nests contained eggs of the same laying order, with similar mass and volume. Furthermore, total clutch mass and total clutch volume of original nests, as measures of maternal quality, were not significantly different among eggs within treatment nests (clutch mass: Friedman $\chi^2 = 1.21$, df = 2, $P = 0.54$; clutch volume: Friedman $\chi^2 = 4.0$, df = 2, $P = 0.13$). Mass and volume of eggs within control nests (i.e., in which laying order = hatching order) remained significantly

different among laying/hatching order groups (mass: Friedman $\chi^2 = 18.0$, df = 2, $P = 0.0001$; volume: Friedman $\chi^2 = 11.6$, df = 2, $P = 0.003$, $n = 27$). Three of the 102 nests were excluded from the study because a-eggs had begun hatching before egg-swapping was initiated.

4.3.2. Hatching, disappearance, and fledging rates

Hatching rates of treatment and control eggs were not significantly different (treatment: 81.9% (177/216), control: 84.0% (68/81), $z = 0.30$, $P = 0.76$). Median hatch spread for control clutches was 3 days (range 2-4 days, $n = 19$ clutches), whereas that for treatment clutches was 4 days (range 2-8 days, $n = 43$ clutches; MWU test: $z = -4.78$, $P < 0.0001$). Median hatching interval between first- and second-hatched chicks was 1 day for control clutches (range 0-2 days, $n = 23$ clutches) and 2 days for treatment clutches (range 0-6 days, $n = 60$ clutches; MWU test: $z = -4.27$, $P < 0.0001$). Median hatching interval between second- and third-hatched chicks was 2 days for both control (range 1-3 days, $n = 20$ clutches) and treatment clutches (range 0-7 days, $n = 45$ clutches; MWU test: $z = -2.13$, $P = 0.033$). Disappearance rates of hatchlings were 7.9% (14/177) and 8.8% (6/68) in treatment and control groups, respectively ($z = 0.17$, $P = 0.86$). Of the chicks with known fates (i.e., dead or fledged), 73.0% (119/163) of treatment chicks fledged, whereas control chicks fledged at a rate of 82.3% (51/62); this difference was not significant ($z = -1.19$, $P = 0.23$, but see below). Median fledging age was 33 days for treatment (95% CL = 33.1 – 34.2 days, $n = 119$) and control groups (95% CL = 32.8 – 34.5 days, $n = 51$). Within the treatment group, median fledging age of third-hatched chicks was 1.5 days later than that of first-hatched chicks (first-hatched chicks: 33 days, $n = 59$, second-hatched chicks: 34 days, $n = 48$, third-hatched chicks: 34.5 days, $n = 12$, KW statistic = 10.14 , $P = 0.006$). This trend was not observed in

the control group (first-hatched chicks: 33 days, n = 18, second-hatched chicks: 33 days, n = 19, third-hatched chicks: 32 days, n = 14, KW statistic = 2.64, P = 0.27).

4.3.3. Effect of laying order and hatching order on survival

Hatching order had a significant effect on chick survival (χ^2 = 7.33, df = 2, n = 225, P = 0.025), whereas laying order did not (χ^2 = 3.05, df = 2, P = 0.22; Figure 4.2). Third-hatched chicks were 60.4 times more likely to die than first-hatched chicks (95% CL = 11.1 – 326.6, z = -4.76, P < 0.0001). The effect of hatching order on survival appeared more dramatic among treatment birds than among control birds (Figure 4.2). Although there was no effect of treatment (χ^2 = 0.62, df =1, P = 0.43), or treatment × hatching order interaction (χ^2 = 4.21, df = 2, P = 0.12), third-hatched treatment chicks were 7.0 times more likely to die than third-hatched control chicks (95% CL = 1.8 – 26.8, χ^2 = 8.14, P = 0.004). There was no significant difference in survival between treatment and control chicks hatching first, (χ^2 = 2.42, P = 0.12), or second (χ^2 = 1.39, P = 0.24). Closer examination of the treatment group revealed no significant difference in survival among chicks from a-, b-, and c-eggs made to hatch first (a-chicks: 19/19, b-chicks: 20/22, c-chicks: 20/21, P = 0.77), second (a-chicks: 17/20, b-chicks: 13/19, c-chicks: 18/21, P = 0.29), or third (a-chicks: 6/17, b-chicks = 3/10, c-chicks: 3/14, P = 0.76).

4.4 Discussion

The cross-fostering design created clutches containing eggs of the same laying order, of similar mass and volume, and from females of similar quality. Hatching order, independent of laying order, had a significant effect on prefledgling survival of Franklin's gulls, whereas laying order had no observable effect. When examining each hatching order category separately, there was no significant difference in survival among chicks hatching from eggs of

different laying sequences. These results suggest that hatching asynchrony exerts a considerably greater influence on nestling survival than do intraclutch differences in egg quality associated with laying order.

Third-hatched chicks experienced significantly higher mortality rates, and slower rates of development (as indicated by older fledging ages) in the treatment group than in the control group. Both observations may have been caused by the longer hatch spread that occurred within treatment clutches. Median hatch spread of treatment nests was one day longer than in the control group, which likely magnified the sibling hierarchy, and exacerbated the third chick disadvantage caused by hatching asynchrony. Another possible explanation is that, by controlling for laying order, there was loss of compensatory effects of c-egg yolk constituents such as androgens which have been hypothesized to offset the negative effects of hatching asynchrony to last-laid chicks (Schwabl 1993; Royle *et al.* 2001). Although the manipulation performed on treatment nests produced a longer hatch spread than that normally observed in nature, the interpretation that hatching asynchrony can override the effects of laying order still holds. There was no difference in survival among chicks placed in similar competitive environments, regardless of what their original position in the laying sequence was, and regardless of the package of materials with which they were originally equipped.

Several studies have shown that hatching asynchrony is more important than egg mass in creating size hierarchies among siblings within a brood (e.g., Figure 4.3), and in causing higher mortality rates in last-hatched chicks (Magrath 1992; Royle & Hamer 1998). The role of intraclutch variation in egg mass for chick survival in asynchronously hatching birds is still unclear (Williams 1994; Bernardo 1996). Whereas some studies have concluded that egg size has profound effects on chick survival (Parsons 1970; Lundberg & Väisänen 1979; Thomas

1983; Bolton 1991; Pelayo & Clark 2003), other studies have found no effect (Bolton *et al.* 1992; Sydeman & Emslie 1992; Styrsky *et al.* 1999; Nager *et al.* 2000; Krist *et al.* 2004). Results from correlative studies examining the relationship between egg size and survival should be interpreted with caution because potentially confounding effects of factors such as parental quality or hatching asynchrony have not been controlled for (Williams 1994; Bernardo 1996; Nager *et al.* 2000). For instance, in a frequently cited study (Parsons 1975a), it was concluded that egg size affected chick survival in addition to the effects of hatching asynchrony in herring gulls (*Larus argentatus*). Pipping a- and c-eggs of different nests had been swapped, resulting in nests with a-eggs hatching last (a- and b-eggs from original nest plus transferred a-egg), and c-eggs hatching first (transferred c-egg plus b- and c-eggs from original nest). Similar to our study, a-chicks made to hatch last had increased mortality, and c-chicks made to hatch first had improved survival. In contrast to our study, mortality of last-hatched a-chicks was lower than that of normal c-chicks, and survival of first-hatched c-chicks was lower than that of normal a-chicks. It was subsequently concluded that the remaining differential mortality was caused by intraclutch differences in egg size; however, female quality, laying order, and egg mass had not been controlled for in their cross-fostering experiment, and egg mass differences within altered broods were not reported, nor were their effects on survival investigated. Differences in female quality (e.g., clutch initiation date or total clutch mass) between nests in which eggs were traded had not been examined; hence, observed differences may have been influenced by among-clutch effects of swapped eggs in addition to within-clutch effects.

Some studies which have controlled for potentially confounding factors such as parental quality demonstrated that egg size had significant effects on chick survival only

within the first week (or less) post-hatch (Bolton 1991; Williams 1994; Nisbet *et al.* 1998; Styrsky *et al.* 1999; Christians 2002). Survival of prefledgling lesser black-backed gulls (*L. fuscus*) raised by foster parents, and in the absence of sibling rivalry, was significantly affected by laying order (Nager *et al.* 2000). This was attributed to changes in yolk composition (i.e., reduced lipid content with laying order), as egg size had no significant effect on survival independent of laying order. It is possible that laying order would have similar effects in Franklin's gull chicks raised singly; however, in the presence of sibling competition, any effect of laying order was overridden by effects of hatching order because neither egg size nor laying order had an effect on Franklin's gull survival to 7 days (Chapter 5) or to fledging (this study; Chapter 5).

If laying order has no observable effect on chick survival over and above the effects of hatching asynchrony, is within-clutch variation in egg quality adaptive, or is it a consequence of the proximate constraints imposed upon females during the egg-laying period? Egg production in *Larus* spp. is energetically and nutritionally demanding (Ricklefs 1974; Robbins 1981; Houston *et al.* 1983; Mawhinney *et al.* 1999), and energy or nutrient constraints significantly affect intraclutch egg size variation (Pierotti & Bellrose 1986; Bolton 1991; Bolton *et al.* 1992). During egg production, female Franklin's gulls produced on average 45% of their body mass in eggs (Soos, unpublished data). Such an investment is similar to that of other gull species in which energy requirements for egg laying rise to 170% of basal metabolic rate (Ricklefs 1974). Proteins (Houston *et al.* 1983; Bolton *et al.* 1992) and carotenoids (Blount *et al.* 2004) have also been shown to limit egg production and size. Hence, it is possible that the apparent differential allocation of resources into eggs within a clutch is a reflection of the increasing energetic and nutritional constraints experienced by the female as

she continues to lay eggs (Houston *et al.* 1983; Pierotti & Bellrose 1986; Sydeman & Emslie 1992; Aparicio 1999; Krist *et al.* 2004).

Although laying order and egg size did not significantly affect chick survival in this study, it cannot be concluded that differences in egg quality associated with laying order have negligible effects on offspring fitness. Firstly, the only components of egg quality measured in this study were egg mass and volume; it is unknown whether levels of yolk constituents of Franklin's gull eggs decline with laying order as has been demonstrated in other *Larus* spp. (e.g., *L. delawarensis*: Meathrel and Ryder 1987; *L. fuscus*: Royle *et al.* 1999 & 2001; Blount *et al.* 2002; *L. ridibundus*: Groothuis and Schwabl 2002). Regardless of the pattern in Franklin's gull eggs, I believe it is reasonable to assume that yolk constituents may have been controlled for with the cross-fostering design because other measures of egg quality (i.e., egg mass and volume) and female quality were successfully controlled for. Secondly, this study cannot rule out the possibility of long-term effects associated with intraclutch variation in egg quality (e.g., effects on recruitment to the breeding population) because only one component of fitness, survival to fledging, was examined. Nonetheless, in addition to chick survival, factors which have the potential to affect fitness, such as prefledgling mass, growth, and cell-mediated immune function (all of which were positively associated with prefledgling survival, Chapter 5), were significantly associated with hatching order, but not with laying order, egg mass, or egg volume (Chapter 5, but see section 5.4.3). Consequently, these results indicate that intraclutch variation in egg quality does not predetermine the fate of prefledgling gulls, and may be less important than hatching asynchrony for offspring fitness in Franklin's gulls. Furthermore, intraclutch variation in egg size is generally outweighed by variation among clutches in numerous species (reviewed by Christians 2004), and may thus have little

consequence for offspring fitness post-fledging, at which time chicks begin interacting with individuals other than their nest mates. Adaptive explanations for the decline in egg quality associated with laying order should therefore be proposed with caution, and require further empirical evidence. A better understanding of the relationships among egg size, egg components, and offspring growth, immune function, survival, and fitness is needed. Experiments controlling for laying order, hatching order, and parental quality, or experiments removing the effects of sibling competition should be incorporated into future studies exploring these relationships.

Figure 4.1. Photographs illustrating nest enclosures employed to facilitate locating chicks, and to prevent them from moving between nests. Enclosures appeared to blend in visually with emergent vegetation (above), and did not prevent adults from incubating eggs or caring for chicks (left).

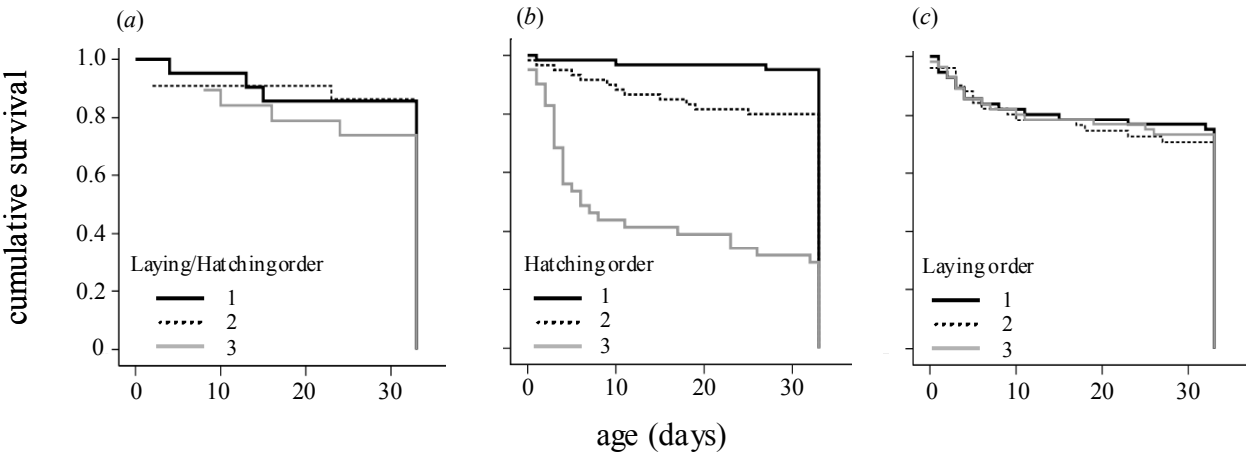

Figure 4.2 Survival curves of Franklin's gull chicks with known fates (i.e., dead or fledged), demonstrating (a) the effects of both laying order and hatching order on survival rates of control chicks (*n* = 62); (b) the effect of hatching order on survival rates of chicks in treatment group, while controlling for laying order, egg size, and female quality (*n* = 163); and (c) the effect of laying order on survival rates of chicks in treatment group, while controlling for effects of hatching order (*n* = 163). Chicks which survived to fledging were assigned a fledging age of 33 days for this analysis.

Figure 4.3. Photograph illustrating the size hierarchy created by hatching asynchrony. Chicks are standing on nest in order of hatch from left to right.

5. UNRAVELLING THE COMPLEXITY OF LIFE HISTORY TRADE-OFFS INVOLVING IMMUNE FUNCTION: THE ROLE OF HATCHING ASYNCHRONY, CONDITION, STRESS, AND STAGE OF DEVELOPMENT[1]

5.1. Introduction

Life history theory predicts that relationships among fitness components within individuals are constrained by limited time, energy, or nutrient resources (Stearns 1992). The immune response has been increasingly explored and recognized as a potentially costly trait that produces trade-offs with other fitness components such as growth and reproduction (Gustafsson *et al.* 1994; Sheldon & Verhulst 1996; Norris & Evans 2000). Involvement of the immune system in physiological trade-offs is evident in studies that have demonstrated depressed growth (Klasing *et al.* 1987; Fair *et al.* 1999; Brommer 2004) or reproductive output (Ilmonen *et al.* 2000; Råberg *et al.* 2000) following upregulation of the immune system, and depressed immune responsiveness following increased reproductive effort (Deerenberg *et al.* 1997; Nordling *et al.* 1998; Ardia *et al.* 2003). Other studies have found no significant correlation (Svensson *et al.* 1998; Williams *et al.* 1999; Hõrak *et al.* 2000; Hõrak *et al.* 2003), resulting in debate regarding the costs of immune function (Svensson *et al.* 1998; Owens & Wilson 1999). There is currently sufficient evidence to support the notion that upregulation of the immune system is costly (Klasing 1998; Lochmiller & Deerenberg 2000; Ots *et al.* 2001; Bonneaud *et al.* 2003; Derting & Compton 2003; Martin *et al.* 2003); hence,

[1] A similar version of this chapter will be submitted for publication as Soos *et al.* (2004b).

an alternate explanation is required to account for the failure to demonstrate trade-offs in some studies.

One possibility is that trade-offs are masked depending on an individual's condition (van Noordwijk & de Jong 1986; Sandland & Minchella 2003). Individuals in excellent body condition may have sufficient resources to successfully undergo multiple energy-costly processes, resulting in a positive correlation between fitness components hypothesized to be competing against each other. Furthermore, trade-offs might not be apparent in emaciated individuals in which resources are likely diverted to maintain core body functions required for survival, and away from growth, reproduction, and immune function. Trade-offs should be most prominent in individuals in moderate to poor body condition, where resources above those required for maintenance are limited (Sandland & Minchella 2003). Resource-dependent trade-offs have been demonstrated in invertebrates (Moret & Schmid-Hempel 2000), but require further exploration in vertebrates.

Another possible explanation for failure to demonstrate trade-offs between immune function and other traits is that trade-offs may change in magnitude during different stages in an individual's life cycle (Sandland & Minchella 2003). For example, the first week of life is a time of high energy demand in birds because of rapid growth rates and development of multiple organ systems (Sheldon & Verhulst 1996); hence, trade-offs (e.g., between growth and immune function) might be most prominent at this stage of development, and less obvious or masked in later stages of development.

Trade-offs also may be significantly affected, or perhaps mediated, by the social environment (Zuk & Johnsen 2000). Immune function, body condition, reproductive success, and survival can be influenced by an individual's dominance status through its effects on the

physiological stress response (Barnard *et al.* 1998), or its effects on resource acquisition, including food, mates, and territories (Zuk & Johnsen 2000). For example, sexual ornamentation and immune response were positively correlated in dominant male red jungle fowl (*Gallus gallus*), and negatively correlated in subordinate males (Zuk & Johnsen 2000). The trade-off experienced by subordinate individuals was not mediated by body condition, as there was no difference in body condition between dominant and subordinate males, but could have been mediated by stress and immunosuppressive endogenous glucocorticoids. In species with hatching asynchrony, trade-offs may be magnified within chicks lower in the age and size hierarchy. Trade-offs in this case would likely be mediated by both condition and the stress response because last-hatched chicks tend to be poorer in body condition (Bollinger *et al.* 1990; Sydeman & Emslie 1992), and likely experience higher stress levels than their siblings. Testosterone may also play a role in modulating immune responses or trade-offs (Duffy *et al.* 2000; Evans *et al.* 2000; Casto *et al.* 2001), though its ultimate effects on immune function are unclear (Braude *et al.* 1999; Hasselquist *et al.* 1999).

I examined relationships among hatching asynchrony, laying order, body condition, corticosterone, testosterone, immune function, growth, and survival at two stages of development in nestling Franklin's gulls. In this study, it was necessary to separate the effects of laying order from hatching order, as egg quality typically varies with laying order in many species, including gulls (Meathrel & Ryder 1987; Magrath 1992). Therefore, I conducted a cross-fostering experiment to create clutches containing asynchronously hatching eggs of similar quality. My objectives were to investigate (i) factors affecting condition, corticosterone levels, and immune function in neonatal chicks, and in chicks that survived to 2 weeks of age, (ii) factors affecting survival during the first week of age, and to fledging, and

(iii) the role of hatching asynchrony in trade-offs among immune responsiveness, growth, and survival in prefledgling gulls.

I predicted that factors influencing immune function and survival would vary at different stages of development because, in an investigation of the proximate causes of mortality in juvenile Franklin's gulls, the most common causes of neonatal mortality (drowning, or trauma from predation or aggression by siblings or other conspecifics) were often associated with starvation, while diseases associated with immunosuppression were common in older juveniles (Appendix D). As laying order effects on immune function and survival should be limited to the neonatal stage (Bolton 1991; Williams 1994), I predicted that effects of hatching order would become increasingly pronounced with development. In addition to having significant effects on condition, growth rate, endogenous hormone levels, immune function, and survival, hatching asynchrony was predicted to affect the existence or magnitude of trade-offs between immune function and other life history traits.

5.2 Methods

5.2.1. Experimental procedures

Detailed methods for cross-fostering are presented in Chapter 4. The cross-fostering design produced 72 treatment and 27 control clutches. Treatment clutches contained eggs of the same laying order, with similar egg mass and volume, and from females of similar quality (Chapter 4). Control clutches contained original eggs laid by the same female; hence, laying order and hatching order were the same. As in Chapter 4, all observations and manipulations were performed from a kayak.

During the hatching period (1-15 June for study nests), each nest was visited daily to determine hatching date and order. To identify individual chicks, the lateral surface of the

right tarsometatarsus or downy feathers under the right wing were marked with coloured indelible ink; coloured plastic leg bands were fitted by 1 to 2 weeks of age. At 0-2 days of age, mass (nearest 0.5 g) and length of tarsometatarsus (TMT, nearest 0.1 mm) were measured, and blood samples were obtained by jugular venipuncture from all hatchlings. Blood was stored in lithium heparin tubes which were placed on ice packs until centrifuged. Plasma was frozen at -20°C until analyzed for corticosterone and testosterone (in ng/ml) using commercial radioimmunoassay kits, as per the manufacturer's instructions (ImmunoChem Double Antibody Corticosterone RIA kit, ICN Biomedicals Inc., Costa Mesa, CA; Coat-A-Count Total Testosterone, Diagnostic Products Corporation, Los Angeles, CA; see also Sorenson *et al.* 2002). Time from arrival at nest-side to blood collection, and chick handling time to blood collection were recorded for all blood samples. The initial stage of the cell-mediated immune (CMI) response of hatchlings was assessed using responses to 40 µg (2 mg/ml solution) phytohemagglutinin (PHA, Sigma-Aldrich, Mississauga, ON) injected in the foot web. Five measurements of foot web thickness (to the nearest 0.01 mm) were obtained prior to and approximately 24 hours after injection using a micrometer. The CMI response was calculated as the change in mean thickness of the PHA-injected site (Smits *et al.* 1999). PHA tests, biometric measurements, and blood collection for hormone analyses were repeated in chicks that survived to 14 days of age. At 14 days, chicks were vaccinated IP with 0.5 ml of a 10% suspension of sheep red blood cells (SRBC, ICN Biomedicals Inc., Aurora, OH, washed twice and resuspended in sterile physiological saline) to induce a primary humoral immune response. Plasma from blood collected 6 days post-vaccination was frozen at -20ºC until tested in duplicate for anti-SRBC antibodies using a standard hemagglutination assay (Smits & Williams 1999). Pre-vaccination titers were also measured, and used to adjust SRBC

responses accordingly. Titres are expressed as the $\log_2$ of the reciprocal of the highest plasma dilution with positive hemagglutination (Lochmiller *et al.* 1993; Smits & Williams 1999). Nests were inspected for mortality every 1-2 days to estimate mortality and fledging dates. Results from gross necropsy and histopathological analyses on prefledgling Franklin's gulls found dead within enclosures are reported in Appendix D.

5.2.2. Statistical analyses

For factors affecting mass, immune function, corticosterone, and survival at two stages of development, multilevel modelling (MLwiN, version 1.1, Multilevel Models Project, Institute of Education, Rasbash *et al.* 2000) was used to account for clustering and random effects of original nest. For model selection, variables with $P < 0.20$ on their own were added to the model together, and removed based on their contribution to the model as well as comparison of AIC values of models, if sample sizes did not change. To minimize problems associated with collinearity, all continuous variables were standardized to have a mean of zero and standard deviation (SD) of 1 (by subtracting each value by its mean and dividing by SD). Highly correlated (>70%) explanatory variables were not included together in models, but examined separately if relevant. Explanatory variables employed to explore factors affecting neonatal mass included group (treatment/control), laying order, hatching order, clutch initiation date, egg mass, egg volume (estimated as per Bolton 1991, see Chapter 4), total original clutch mass, brood size at hatch, corticosterone, and testosterone. Factors examined for neonatal CMI response and corticosterone level included all of the above variables as well as neonatal mass, and TMT length (and corticosterone for CMI response).

Similar models were created to examine factors affecting mass, immune function (both CMI and humoral immunity), and corticosterone at 2 weeks, as well as instantaneous growth

rate (IGR, estimated using $(\log_{10}W_2 - \log_{10}W_1)/t$, where W_1 = neonatal mass, W_2 = mass at 2 weeks, and t = days between measurements, Barbato 1992, Nager *et al.* 2000). Factors explored in these models included all neonatal factors and measurements obtained at 2 weeks of age where relevant. For humoral immunity, additional explanatory variables included growth rate between 2 and 3 weeks (estimated using $(W_3 - W_2)/t$) and rate of size increase from 2 to 3 weeks (as calculated by $(l_3 - l_2)/t$, where l = length of TMT). As treatment and control groups showed similar trends in hatching, disappearance, and fledging rates, (Chapter 4), the two groups were combined in all analyses. Group was included in all models to account or control for its effects. Relevant interactions also were explored.

Binary logistic regression was employed using random intercept models to assess factors affecting survival to 7 days and to fledging, while accounting for clustering at the level of original nest. Model selection and exclusion of variables were performed as for continuous outcome variables, however, analyses were performed in STATA (StataCorp 2003), using maximum likelihood methods to generate estimates (GLLAMM, Rabe-Hesketh *et al.* 2004). Odds ratios (OR) were calculated using exp(b).

Using simple linear regression, plasma corticosterone increased significantly albeit weakly with minutes handling time of neonates ($R^2 = 0.026$, df = 203, $P = 0.025$), and by minutes at nest-side in 2-week-olds ($R^2 = 0.056$, df = 180, $P = 0.001$). To control for these potentially confounding effects, residuals of corticosterone on minutes handling or at nest-side for samples collected from neonates and 2-week-olds, respectively, were employed in all analyses after being square-root transformed (following the addition of 1.5 to correct for all negative values) to give rise to normal distributions. Testosterone was employed as a categorical variable because of the large positive skew observed in its frequency distribution.

Values were assigned into three categories: zero (0 ng/ml), low (0.01-0.10 ng/ml), and high (>0.10 ng/ml).

5.3 Results

5.3.1. Factors affecting neonatal mass, corticosterone, and immune function

Neonatal mass was negatively associated with hatching order, and positively associated with total clutch mass (Table 5.1, model 1). Candidate models using either egg mass or egg volume in lieu of total clutch mass were also significant, but had higher AICs than model 1 (e.g., Appendix E, model E1, AIC = 605.01 compared to 597.64 for model 1, Appendix F). The significance of both egg mass and volume was lost when either was included with total clutch mass (e.g., Appendix E, model E2), possibly due to the strong correlation between egg mass or volume and total clutch mass ($R_{egg\ mass} = 0.85$, $n = 306$, $P < 0.0001$; $R_{egg\ vol} = 0.79$; $n = 306$, $P < 0.0001$), suggesting that total clutch mass (as an index of biological female quality) is a slightly better predictor for neonatal mass than either egg mass or volume. Laying order had a significant effect on neonatal mass when examined alone ($b_{LO2\text{-}LO1} = -0.255$, SE = 0.148, $\chi^2 = 2.963$, $P = 0.085$; $b_{LO3\text{-}LO1} = -0.296$, SE = 0.147, $\chi^2 = 4.084$, $P = 0.043$; $n = 226$). This effect was lost or masked when hatching order was added to the model (Appendix E, model E3).

Neonatal CMI response was significantly affected by hatching order (with third-hatched chicks having the highest response), and negatively associated with neonatal mass (Table 5.1, model 2). The significance of neonatal mass when examined alone ($b = -0.163$, SE = 0.066, $\chi^2 = 6.158$, $P = 0.013$) was reduced when in combination with hatching order in model 2, likely due to the effect of hatching order on mass (see model 1). Removing neonatal mass from model 2 produced a higher AIC (Appendix E, model E4, AIC = 630.15, from

614.51 for model 2, Appendix F), so it was kept in the model. Laying order ($b_{LO2\text{-}LO1}$ = 0.002, SE = 0.147, $\chi^2 < 0.0001$, $P = 1.00$; $b_{LO3\text{-}LO1}$ = 0.241, SE = 0.147, $\chi^2 = 2.691$, $P = 0.101$; n = 225), egg mass (b = -0.044, SE = 0.070, $\chi^2 = 0.401$, $n = 225$, $P = 0.527$), and egg volume (b = -0.079, SE = 0.070, $\chi^2 = 1.305$, $n = 225$, $P = 0.253$) were not significantly associated with neonatal CMI response. Corticosterone had no association with neonatal CMI response when examined alone ($b = 0.040$, SE = 0.069, $\chi^2 = 0.336$, $n = 200$, $P = 0.562$). However, neonatal corticosterone was highest in third-laid, third-hatched chicks (Table 5.1, model 3), within which there was a significant association between corticosterone and CMI when alone in a model ($b_{LO3\cdot HO3} = 0.399$, SE = 0.175, $\chi^2 = 5.213$, $P = 0.022$; reference = all other chicks combined: $b_{all\ other\ chicks}$ = -0.032, SE = 0.075, $\chi^2 = 0.180$, $P = 0.671$; $n = 200$), and when added to model 2 (Appendix E, model E5). With the addition of the latter parameters to model 2, there was a substantial decrease in sample size (n = 197 from 222), so AICs could not be compared. Sample size decreased because a sufficient volume of plasma to adequately measure both corticosterone and testosterone was not obtained from all hatchlings due to the limited volume of blood that could be sampled safely from them at that age/size.

5.3.2. Factors affecting mass, growth rate, corticosterone, and immune function at 2 weeks of age

Mass at 2 weeks of age was significantly affected by hatching order, positively associated with neonatal mass, and negatively associated with neonatal CMI and corticosterone level at 2 weeks (Table 5.2, model 4). Positive trends observed with egg mass ($b = 0.134$, SE = 0.073, $\chi^2 = 3.370$, $n = 183$, $P = 0.066$) and volume ($b = 0.129$, SE = 0.075, $\chi^2 = 2.956$, $n = 183$, $P = 0.086$) disappeared when each variable was included with neonatal mass in models (results not shown). IGR to 2 weeks was significantly correlated with mass at 2

weeks ($R = 0.72$, $n = 179$, $P < 0.0001$), mainly because the former was calculated using the latter; hence, models 4 and 5 are similar (Table 5.2). However, clutch initiation date, which was not significant in any model created for mass at 2 weeks (results not shown), was negatively associated with IGR (Table 5.2, model 5).

Corticosterone level at 2 weeks of age was positively associated with neonatal corticosterone, and, as in model 4, negatively associated with mass at 2 weeks (Table 5.2, model 6). Furthermore, chicks in brood sizes of two had significantly higher corticosterone levels than chicks in brood sizes of three. In a candidate model including hatching order categories in lieu of mass at 2 weeks, corticosterone was positively associated with hatching order, with third-hatched chicks having the highest corticosterone levels (see Appendix E, model E6); however, AIC for this model was higher than that for model 6 (AIC = 448 from 440, Appendix F). When both mass at 2 weeks and hatching order were included in the same model, the significance of hatching order was lost (Appendix E, model E7), likely due to the relationship between hatching order and mass at 2 weeks (see model 4), suggesting that mass at 2 weeks is a better predictor of corticosterone levels than is hatching order.

CMI response at 2 weeks of age was negatively associated with clutch initiation date, and positively associated with IGR only for third-hatched chicks (Table 5.2, model 7). Effects of hatching order were important primarily through interaction with IGR, but not directly. IGR had no association with CMI response of senior chicks ($b_{HO1 \times IGR} = -0.119$, SE = 0.157, $\chi^2 = 0.575$, $P = 0.448$; $b_{HO2 \times IGR} = 0.078$, SE = 0.124, $\chi^2 = 0.395$, $P = 0.530$, from model 7). A candidate model including mass at 2 weeks in lieu of growth rate had similar results (Appendix E, model E8). AICs for the two models could not be compared due to the difference in sample size, but each model was the best in its family of models (Appendix F);

hence, both are potentially acceptable. CMI response was not associated with laying order ($b_{LO2\text{-}LO1}$ = -0.100, SE = 0.173, χ^2 = 0.336, P = 0.562; $b_{LO3\text{-}LO1}$ = 0.100, SE = 0.173, χ^2 = 0.330, P = 0.566; n = 181), egg mass (b = 0.077, SE = 0.074, χ^2 = 1.086, n = 181, P = 0.297), or egg volume (b = 0.095, SE = 0.076, χ^2 = 1.550, n = 181, P = 0.213).

Humoral immune response to SRBC at 3 weeks was significantly affected by laying order, negatively associated with neonatal testosterone, corticosterone at 2 weeks, and clutch initiation date, and positively associated with rate of increase of TMT length from 2 to 3 weeks of age (Table 5.2, model 8). Chicks from second-laid eggs had significantly higher SRBC responses than did chicks from first-laid eggs (Table 5.2, model 8), but there was no difference between responses of chicks from second and third-laid eggs ($b_{LO3\text{-}LO2}$ = 0.121, SE = 0.173, χ^2 = 0.487, P = 0.485). Although egg mass had no association with humoral immune response when examined alone (b = 0.048, SE = 0.077, χ^2 = 0.391, n = 178, P = 0.532), egg volume had a nearly significant positive association (b = 0.138, SE = 0.078, χ^2 = 3.119, n = 178, P = 0.077). This trend was weakened when egg volume was added to model 8, possibly due to the relationship of egg volume and laying order, where second-laid eggs are the largest (outcome variable = egg volume, $b_{LO2\text{-}LO1}$ = 0.296, SE = 0.091, χ^2 = 10.621, P = 0.001; $b_{LO3\text{-}LO1}$ = -0.288, SE = 0.091, χ^2 = 10.047, P = 0.002; n = 306). Neither IGR for the first 2 weeks (b = 0.022, SE = 0.078, χ^2 = 0.077, n = 174, P = 0.781), nor linear growth rate from 2 to 3 weeks (b = 0.064, SE = 0.077, χ^2 = 0.681, n = 177, P = 0.409) had significant associations with humoral immune response when examined alone. Despite its effects on CMI responses, hatching order had no significant effect on humoral immune response ($b_{HO2\text{-}HO1}$ = -0.061, SE = 0.177, χ^2 = 0.120, P = 0.729; $b_{HO3\text{-}HO1}$ = -0.047, SE = 0.214, χ^2 = 0.048, P = 0.827; n = 178).

5.3.3. Factors affecting survival to 7 days of age

The likelihood of mortality occurring within 7 days of age was significantly affected by hatching order, and negatively associated with neonatal mass (Table 5.3, model 9). Third-hatched chicks were 25.2 (95% CL = 2.7 – 237.5) and 15.6 (95% CL = 2.7 – 89.8, $P = 0.002$) times more likely to die within the first 7 days than were first- and second-hatched chicks, respectively (Table 5.3, model 9). Neonatal CMI response was positively associated with the likelihood of mortality within the first week when examined alone ($b = 0.802$, SE = 0.331, OR = 2.2, 95% CL = 1.2 – 4.3, $z = 2.42$, $n = 210$, $P = 0.015$); this relationship was lost when included in models with hatching order, neonatal mass, or both, although a positive trend was still observed (e.g., Appendix E, model E9). The loss of significance was possibly due to the relationship of CMI with both of those covariates (see model 2). Fate before 7 days was also positively associated with corticosterone ($b = 0.804$, SE = 0.369, OR = 2.2, 95% CL = 1.1 – 4.6, $z = 2.18$, $n = 192$, $P = 0.029$). When added to model 9, the trend was no longer significant (Appendix E, model E10). Laying order ($b_{LO2\text{-}LO1} = 0.599$, SE = 0.633, OR = 1.8, 95% CL = 0.53 – 6.3, $z = 0.95$, $P = 0.344$; $b_{LO3\text{-}LO1} = 0.209$, SE = 0.628, OR = 1.2, 95% CL = 0.36 – 4.2, $z = 0.33$, $P = 0.739$; $n = 229$), egg mass ($b = 0.578$, SE = 0.383, OR = 1.8, 95% CL = 0.84 – 3.8, $z = 1.51$, $n = 229$, $P = 0.131$), and egg volume ($b = 0.457$, SE = 0.321, OR = 1.6, 95% CL = 0.84 – 3.0, $z = 1.43$, $n = 229$, $P = 0.154$) were not significantly associated with chick survival to 7 days of age when examined alone.

5.3.4. Factors affecting survival to fledging

The likelihood of mortality occurring between 7 days and fledging date was affected by hatching order, and decreased with mass at 2 weeks and neonatal CMI (Table 5.3, model 10). Of the chicks that survived the first week, third-hatched chicks were 31.9 (95% CL = 2.8

– 363.7) and 8.0 (95% CL = 1.5 – 43.1, $P = 0.016$) times more likely to die before fledging than were first and second-hatched chicks, respectively (Table 5.3, model 10). In a candidate model (Appendix E, model E11), both neonatal mass and IGR were negatively associated with the likelihood of mortality. AICs for the two models could not be compared due to different sample sizes; however, each model was the best in its family (Appendix F). Model 10 was favoured because it had fewer parameters and was, thus, simpler. The likelihood of mortality prior to fledging was nearly significantly associated with CMI response at 2 weeks when examined alone ($b = -0.818$, SE = 0.452, OR = 0.44, 95% CL = 0.18 – 1.1, $z = -1.81$, $n = 179$, $P = 0.071$), but not with humoral immune response ($b = -0.137$, SE = 0.454, OR = 0.87, 95% CL = 0.36 – 2.1, $z = -0.30$, $n = 177$, $P = 0.762$). Trends observed with laying order ($b_{LO2\text{-}LO1} = 0.397$, SE = 0.787, OR = 1.5, 95% CL = 0.32 – 6.7, $z = 0.51$, $P = 0.613$; $b_{LO3\text{-}LO1} = 1.228$, SE = 0.815, OR = 3.41, 95% CL = 0.69 – 16.9, $z = 1.51$, $P = 0.132$; $n = 198$), egg mass ($b = -0.720$, SE = 0.380, OR = 0.49, 95% CL = 0.23 – 1.0, $z = -1.89$, $n = 198$, $P = 0.058$), and egg volume ($b = -0535$, SE = 0.343, OR = 0.59, 95% CL = 0.30 – 1.1, $z = -1.56$, $n = 198$, $P = 0.118$) were reduced when each of those variables was included with neonatal mass in models (e.g., model with egg volume and neonatal mass: $b_{egg\ vol} = -0.401$, SE = 0.408, OR = 0.67, 95% CL = 0.30 – 1.5, $z = -0.98$, $P = 0.325$; $b_{neonatal\ mass} = -1.577$, SE = 0.644, OR = 0.21, 95% CL = 0.06 – 0.73, $z = -2.45$, $n = 187$, $P = 0.014$).

5.4 Discussion

This study illustrates (i) the multifactorial and complex nature of relationships among condition, growth, corticosterone, immune function, and survival (Figure 5.1), (ii) how these relationships are mediated by hatching asynchrony and change with stage of development, (iii) how life history trade-offs among cell-mediated immune function, growth, and survival are

condition-dependent and affected by stage of development, and (iv) the importance of examining both cell-mediated and humoral immunity in studies of immune function-life history trade-offs[2].

5.4.1. Hatching asynchrony, stress, and cell-mediated immunity

Hatching asynchrony is a major driving force shaping the lives of Franklin's gulls, not only because it significantly affected both early and late chick survival, but also because it directly or indirectly affected mass, corticosterone levels, and cell-mediated immune responses at two stages of nestling development (Figure 5.1). Hatching asynchrony also appeared to play a key role in mediating life history trade-offs among cell-mediated immune function, growth, and survival, which will be further discussed below.

There is increasing evidence that acute mild to moderate stress is immunostimulatory and chronic stress is immunosuppressive, and that physiological mechanisms are largely mediated by both the sympathetic nervous system and the hypothalamic-pituitary-adrenal axis (Dhabhar & McEwen 1997; Dhabhar & McEwen 1999; Dhabhar 2000; Edgar *et al.* 2003; Moynihan 2003; Silberman *et al.* 2003). Neonatal CMI response was negatively associated with mass at hatch, and was highest in third-hatched chicks, possibly a result of the acute stress and food-deprivation experienced by last-hatched chicks which must immediately compete with two larger nest mates for food regurgitated by parents. Acute stress is thought to be an adaptive response, and enhances both T-cell dependent humoral

[2] Condition is a term frequently used but uncommonly defined in life-history studies, and usually refers to the amount of energy stores in the body relative to body size (Green 2001). I chose not to employ ratio methods (simple ratios between mass and a linear measure of body size) or mass/length residuals as indices of body condition due to potential problems associated with these techniques (reviewed in Jakob *et al.* 1996 and Green 2001). Rather, I employed mass alone in all analyses as chicks were weighed at the same age, and initial analyses showed mass to be a better predictor than TMT or head-to-bill length when combined in analyses of CMI responses and survival. Hence, a major assumption for the purpose of this discussion is that mass as well as growth rate (when controlling for age) were good indicators of chick condition.

immunity (Silberman *et al.* 2003) and CMI (Dhabhar & McEwen 1997; Dhabhar 2003), the latter by causing a redistribution of lymphocytes from circulation to areas such as skin, lymph nodes, or other vital organs that are high at risk to pathogen entry or trauma. Enhancement of skin immunity during acute stress is mediated by adrenal hormones, i.e., catecholamines and corticosterone (Dhabhar & McEwen 1997; Dhabhar 2003). I did not measure catecholamine levels, but it is possible that corticosterone played some role in mediating CMI responses of neonatal third-hatched chicks, because there was a significant positive relationship between corticosterone and CMI response of chicks that were third-laid and third-hatched. Third-laid, third-hatched chicks had the highest levels of corticosterone, possibly resulting from a combination of the variation in yolk corticosterone levels among eggs within original clutches (e.g., see Hayward & Wingfield 2004), and the effects of acute stress experienced by third-hatched chicks (Nuñez de la Mora *et al.* 1996; Tarlow *et al.* 2001).

Corticosterone at 2 weeks was positively associated with neonatal corticosterone, suggesting that the stressors experienced at hatch had persisted, resulting in chronic stress. Corticosterone was negatively associated with mass at 2 weeks, suggesting that stress is energetically costly. However, the direction of causality of this relationship is unknown as the two measurements were obtained at the same time; hence, it is equally plausible that smaller chicks with slower growth rates were more stressed as a result of being subordinate and/or food-deprived, resulting in higher corticosterone levels. Although it is possible that both scenarios were occurring simultaneously (as one could trigger the other), the latter explanation is supported by evidence that corticosterone levels at 2 weeks of age increased with hatching order, suggesting that the association of mass at 2 weeks with corticosterone was mediated by the effect of hatching order on mass. Third-hatched chicks that survived to 2 weeks were the

smallest, and had the slowest growth rates and highest corticosterone levels, suggesting that they were the most food-deprived and stressed. Third-hatched chicks also had the lowest CMI responses at 2 weeks, although this trend was not significant. Chronic stress associated with prolonged sibling rivalry may have been responsible for this trend; however, there was no negative association between corticosterone and CMI at 2 weeks. This lack of an association might be explained by the recent finding that chronic stress increases the sensitivity of T-lymphocytes to the inhibitory effects of stress hormones (corticosterone and catecholamines), and that diminished immune responses associated with chronic stress may be related to T-lymphocyte receptor density rather than the actual concentrations of the stress hormones themselves (Edgar *et al.* 2003; Silberman *et al.* 2003; Silberman *et al.* 2004). Alternatively, changes in CMI response associated with hatching order may have been mediated primarily through body condition (discussed below).

5.4.2. Relationships among cell-mediated immunity, growth, and survival: The role of hatching asynchrony and condition

The higher CMI responses observed in smaller or third-hatched neonates appeared to have significant repercussions on subsequent growth rate. Mass at 2 weeks and growth rate to 2 weeks were negatively associated with neonatal CMI response, suggesting a trade-off between activation of cell-mediated immune responses and growth at the newly hatched stage, when growth rates are rapid and exponential (Cogburn *et al.* 2000). During the first 2 weeks of life, nestlings increased their body mass on average 5.8 fold from hatch (SD = 1.2; range = 2.0 – 8.7, n = 85; Soos, unpublished data). Resources required to support a high CMI response may be diverted from growth during this stage, when resources have the potential to become limited.

The repercussions of high neonatal CMI responses and stress (i.e., corticosterone level) on survival within the first week are less clear. Both neonatal CMI response and corticosterone level were negatively associated with early survival when each was examined alone in models. However, their significance diminished when included in models with neonatal mass and hatching order, suggesting that these latter two variables were better predictors for survival, with little, if any, additional effect of CMI function or corticosterone level. Hatching order cannot affect survival as a factor on its own, but must act through its effects on other traits such as neonatal mass, CMI, and corticosterone. The importance of the latter two variables for survival may have been masked as a consequence of their relationship with hatching order and mass (CMI only) which remained in the model. The predominant factors threatening survival at this stage of development are aggressive sibling interactions and food-deprivation, thus, body condition (i.e., mass) and hatching order, through their effects on chick competitive ability, are likely to dictate whether a chick will survive sibling interactions and avoid starving to death. Most chicks that died within the first week were third-hatched chicks in poor body condition (Chapter 3, Appendix D). The main causes of death were starvation, trauma from sibling aggression, and drowning (Appendix D), and regardless of the immediate cause of death, 67% (18/27) of chicks that died within the first week were emaciated. It is reasonable to speculate that chicks in poor body condition or on the verge of starvation could have been pushed over the edge if resources for maintenance required for survival were diverted to stress or vigourous immune responses. Further investigation is required to decipher whether the positive association between neonatal CMI response and early survival was a causal relationship, or a correlation resulting from the condition-dependence of both immune function and survival.

Although growth rate and subsequent mass appeared to be negatively affected by high CMI responses early in life, high growth rates were not associated with depressed CMI responses at 2 weeks. Instead, CMI response at 2 weeks was positively associated with growth rate or mass at 2 weeks, but only in third-hatched chicks. Third-hatched chicks had significantly lower mass and growth rates compared to senior chicks; hence, it is likely that they did not have sufficient resources to simultaneously invest in growth and CMI responses (as well as the energy associated with increased stress). Whereas CMI response appeared to be condition-dependent in third-hatched chicks, a similar relationship was not observed in senior chicks, possibly because these chicks were in good condition. Once an animal is in good condition, further increases in condition might not result in higher immune responses beyond some optimal or maximum level, either because higher responses are pathological (Zuk & Stoehr 2002), or because there is a limit to the number of lymphocytes that could be called to the site of injection.

Survival to fledging was positively associated with mass at 2 weeks and growth rate, and negatively associated with hatching order. Neonatal CMI response, which appeared to negatively affect subsequent growth rate, was positively associated with survival to fledging in chicks that managed to survive the first week of life. A high CMI response in neonates poor in body condition could potentially come at a high cost (i.e., death due to starvation). However, in neonates that had sufficient resources to survive past one week of age and maintain high growth rates, high CMI responses appeared to significantly increase survival to fledging, despite the trade-off that occurred between neonatal CMI response and growth. This suggests that trade-offs observed among CMI, growth, and survival were condition-dependent, and that chicks of different quality paid different costs to maintain growth and immune

function simultaneously. Costs appeared to be lower for chicks in good body condition (e.g., first-hatched chicks), and higher for chicks in poor body condition (e.g., third-hatched chicks).

A handful of studies have examined trade-offs involving immune function during chick development. CMI response of 8-day-old great tits (*Parus major*) was positively correlated with subsequent growth rate, and it was suggested that trade-offs between immune function and growth result through competition for specific nutrients such as carotenoids, rather than for energy resources (Hõrak *et al.* 2000). However, positive correlations between traits that are linked in a functional trade-off can result if there are sufficient resources, regardless of currency, to perform both activities successfully (van Noordwijk & de Jong 1986; Sandland & Minchella 2003). It is when resources are limited that trade-offs might become obvious. Brommer (2004) and Soler *et al.* (2003) determined that nestling blue tits (*Parus caeruleus*) and magpies (*Pica pica*), respectively, supplemented with dietary methionine (a T-cell immunostimulant) had higher responses to PHA injections and lower growth rates than untreated controls, and this was attributed to the trade-off between immune function and growth. Despite their lower growth rate, methionine supplemented magpies were less parasitized, and ended up with similar fledging weights to control birds, suggesting that subsequent growth rates of untreated birds had slowed due to parasitism or disease (Soler *et al.* 2003). Captive Japanese quail chicks vaccinated with SRBC, *Mycoplasma synoviae*, and Newcastle disease virus antigens had reduced growth rate and wing length compared to controls, but the depression of growth was transient, and there was no difference in mass or wing length between treatment and controls by 54 days of age (Fair *et al.* 1999). In my study, it is possible that the cost of reduced growth subsequent to high neonatal CMI responses may

have also been overcome or compensated for by reducing costs associated with parasitism or disease, thus leading to increased survival to fledging.

5.4.3. Humoral immunity – Driven by different mechanisms?

Primary humoral immune response measured at 3 weeks was negatively associated with neonatal testosterone and corticosterone level at 2 weeks. Although the concept of immunosuppression by testosterone appears to be widely accepted in the behavioural ecology literature (Braude *et al.* 1999), testosterone has been found to have positive (Evans *et al.* 2000), negative (Duffy *et al.* 2000; Casto *et al.* 2001), or no effects (Hasselquist *et al.* 1999) on immune function of adult birds, usually males. The mechanism by which testosterone is immunosuppressive is unclear, and may be mediated through corticosterone which is often elevated in conjunction with testosterone (Ketterson & Nolan 1992; Duffy *et al.* 2000; Poiani *et al.* 2000; Casto *et al.* 2001; Buchanan *et al.* 2003). In the gull chicks, neonatal testosterone was associated with depressed humoral immune response, in addition to the negative effects of corticosterone level at 2 weeks. Possible sources of testosterone in neonates include residual testosterone from yolk, as well as gonadal testosterone produced by male chicks, or by chicks during aggressive interactions (Groothuis 1989; Groothuis & Meeuwissen 1992). Contrary to our study, testosterone implants in 9-day-old black-headed gulls (*L. ridibundus*) enhanced primary responses to SRBC administered at 2 weeks of age (Ros *et al.* 1997). However, similar to our results, this effect lasted long after testosterone implants were removed (>1 month after removal), suggesting that plasma testosterone level in early developmental stages has long-term effects on humoral immunity.

In contrast to CMI responses, SRBC responses were not associated with hatching order, mass, growth rate, or survival. This does not necessarily suggest that humoral

immunity had no effect on post-fledging survival or fitness, nor that it lacked condition-dependence. SRBC response was positively associated with growth in leg length during the time it took for chicks to develop antibodies to SRBC. Furthermore, corticosterone at 2 weeks, which was negatively associated with SRBC response, was significantly associated with both hatching order and mass at 2 weeks, suggesting that condition may have played some role, if indirect, in SRBC responses. The apparent lack of condition-dependence also may have been a function of the costs associated with developing a primary (IgM) response to a benign antigen. Primary responses to SRBC vaccination in captive northern bobwhite chicks (*Colinus virginianus*) were not affected by protein-deficient diets, whereas PHA responses were significantly depressed, and positively correlated with growth rate and mass (Lochmiller *et al.* 1993). It was suggested that primary responses may not be as dependent on a fully-developed bursa of Fabricius as are secondary (IgG) responses (as per Toivanen *et al.* 1987). Secondary responses involve a significantly higher magnitude of antibody production and upregulation of the humoral immune system (Tizard 1992), and likely pose a substantially higher cost than responses at initial exposure. Severe dietary protein restriction in poultry chicks significantly affected secondary, but not primary, responses to SRBC (Glick *et al.* 1981). Despite its increasingly widespread use in studies of immune function-life history trade-offs, the primary response to vaccination may not be an adequate measure when evaluating the costs associated with humoral immunity. Secondary immune responses might be more appropriate in such studies.

Most studies investigating immune function in relation to other life history components have used a single measure of immune function. As seen in this study, different arms of the immune system may not respond similarly to trade-offs or environmental

conditions; hence, a resource trade-off between immune function and some other life history trait may be overlooked if only one test is employed (Zuk & Stoehr 2002).

5.4.4. Role of laying order, egg size, and female quality

Laying order had a significant effect on SRBC responses, as chicks from second-laid eggs had higher responses than chicks from first-laid eggs. This may have been partially due to the relationship between laying order and egg volume, because second-laid eggs were the largest eggs in clutches. Egg volume may be a reflection of egg quality, and has been positively associated with yolk size, albumen, carotenoids, or lipids (Parsons 1979; Meathrel & Ryder 1987; Royle *et al.* 2001); hence, it is possible that improved SRBC response in chicks from second-laid eggs was related to some component(s) of egg quality.

Effects of laying order and egg size on CMI response and early survival were overridden by effects of hatching order. This was somewhat surprising because other studies have shown that egg size significantly affected early survival (Bolton 1991; Williams 1994; Nisbet *et al.* 1998; Styrsky *et al.* 1999). In my study, the importance of egg mass was limited to its effects on neonatal mass which, in turn, was directly associated with both CMI response and early survival in final models. Despite the relationship between egg mass and neonatal mass, total clutch mass, a measure of female quality, appeared to be a better predictor of neonatal mass than were egg mass or volume. This is likely due to the strong correlation between total clutch mass and egg mass; females in better body condition are more likely to lay larger eggs, resulting in larger total clutch mass compared to females in poorer body condition (Bernardo 1996).

Clutch initiation date was employed as another measure of female quality, and was negatively associated with growth rate to 2 weeks, CMI response at 2 weeks, and humoral

response measured at 3 weeks, of which the former was directly associated with survival to fledging. Offspring production and recruitment are generally higher for females that nest earlier in many orders of birds (Brown & Brown 1999). This may be related to individual female quality, or a reduction in food availability as the season progresses (Brown & Brown 1999). As all clutches employed in this study were initiated within an 8 day period, food availability was unlikely to have differed greatly among clutches. In Franklin's gulls, total clutch mass declined significantly with clutch initiation date (Soos, unpublished data); thus, in this study, clutch initiation date was likely an indicator of individual female quality. In other gull species, females breeding earlier tend to be older, more experienced, or in better body condition than later breeders (Parsons 1975b; Sydeman *et al.* 1991). Neonatal mass, growth rate to 2 weeks, and both arms of the immune system after 2 weeks appeared to be affected by quality of mother in addition to sibling interactions, stress, condition, and hatching or laying order, illustrating the complexity of relationships that define these traits, and ultimately survival to fledging.

5.5. Conclusions

The relationships among fitness components including immune function are very complex; whether and how they trade off with each other physiologically will depend on a variety of factors (Zuk & Stoehr 2002), including the species, population, environmental conditions, social interactions, and body condition of individuals. Whether or not studies are successful in detecting these trade-offs will depend on the type of immune challenge employed, component(s) of the immune system challenged or measured, life history trait(s) measured, and whether or not the study was performed in captive (i.e., *ad libitum*-fed) or free-ranging animals.

Much of this discussion is based upon interpretations of observations and statistically significant associations. However, I believe this study is a first step towards using a multifactorial and multidisciplinary approach to demonstrate interactions and potential trade-offs among life history traits, the physiological mechanisms that produce these relationships, and how these relationships may change depending on stage of development. This study illustrates how life history traits are the outcome of interactions among energy metabolism, endocrine control mechanisms, and the immune system in response to different environmental challenges (Wikelski & Ricklefs 2001).

Table 5.1. Models selected for factors affecting mass, CMI responses, and corticosterone levels in Franklin's gull hatchlings tested at 0-2 days of age.

Variable[a]	*b*	SE	χ^2	*P*	*n*
Model 1 – Neonatal mass					226
Constant	0.406	0.140			
Tx (ref = con)	-0.218	0.133	2.693	0.101	
HO2 (ref = HO1)	-0.318	0.141	5.124	0.024	
HO3 (ref = HO1)	-0.619	0.151	16.821	<0.0001	
Total clutch mass	0.337	0.059	33.044	<0.0001	
Model 2 – Neonatal CMI					222
Constant	-0.261	0.164			
Tx (ref = con)	0.060	0.157	0.146	0.702	
HO2 (ref = HO1)	0.222	0.159	1.960	0.162	
HO3 (ref = HO1)	0.512	0.175	8.581	0.003	
Neonatal mass	-0.112	0.067	2.752	0.097	
Model 3 – Neonatal CORT					204
Constant	-0.065	0.076			
HO3·LO3	0.446	0.197	5.152	0.023	

a. ref = reference category for categorical variable; con = control group; tx = treatment group; HO = hatching order; LO = laying order; CMI = cell-mediated immune response as measured by PHA (phytohaemagglutinin) skin test; CORT = corticoserone

Table 5.2. Models selected for factors affecting mass, IGR, corticosterone level, CMI response at 2 weeks, and humoral immune response measured at 3 weeks in prefledgling Franklin's gulls.

Variable[a]	*b*	SE	χ^2	*P*	*n*
Model 4 – Mass at 2 weeks					174
Constant	-0.121	0.156			
Tx (ref = con)	0.282	0.145	3.755	0.053	
HO2 (ref = HO1)	-0.099	0.145	0.467	0.494	
HO3 (ref = HO1)	-0.492	0.192	6.557	0.010	
Neonatal CMI	-0.154	0.067	5.295	0.021	
Neonatal mass	0.309	0.066	21.966	<0.0001	
CORT at 2 weeks	-0.273	0.066	17.197	<0.0001	
Model 5 – IGR to 2 weeks					174
Constant	-0.092	0.170			
Tx (ref = con)	0.307	0.152	4.063	0.044	
HO2 (ref = HO1)	-0.066	0.154	0.182	0.670	
HO3 (ref = HO1)	-0.775	0.209	13.722	0.0002	
Neonatal CMI	-0.157	0.067	5.429	0.020	
CORT at 2 weeks	-0.259	0.066	15.287	0.0001	
CID	-0.179	0.084	4.572	0.033	
Model 6 – CORT at 2 weeks					158
Constant	-0.150	0.138			
Tx (ref = con)	0.127	0.167	0.580	0.446	
BS1 (ref = BS3)	0.477	0.480	0.987	0.320	
BS2 (ref = BS3)	0.357	0.183	3.823	0.051	
Neonatal CORT	0.149	0.078	3.637	0.057	
Mass at 2 weeks	-0.285	0.076	14.020	<0.0001	
Model 7 – CMI at 2 weeks					177
Constant	-0.178	0.182			
Tx (ref = con)	0.339	0.170	3.972	0.046	
HO2 (ref = HO1)	-0.231	0.171	1.824	0.177	
HO3 (ref = HO1)	0.281	0.247	1.288	0.256	
CID	-0.300	0.092	10.523	0.001	
IGR×HO3	0.334	0.133	6.313	0.012	
IGR×HO1 (ref = IGR×HO3)	-0.453	0.204	4.925	0.026	
IGR×HO2 (ref = IGR×HO3)	-0.256	0.181	1.992	0.158	
Model 8 – Humoral response at 3 weeks (vaccinated at 2 weeks)					144
Constant	0.170	0.221			
Tx (ref = con)	0.069	0.179	0.150	0.699	
LO2 (ref = LO1)	0.394	0.167	5.541	0.019	
LO3 (ref = LO1)	0.273	0.169	2.612	0.106	
CORT at 2wks	-0.166	0.071	5.414	0.020	
Neonatal testosterone – low (ref = zero)	-0.495	0.179	7.656	0.006	
Neonatal testosterone – high (ref = zero)	-0.950	0.292	10.586	0.001	
CID	-0.245	0.098	6.297	0.012	
Skeletal growth (TMT) from 2 to 3wks	0.174	0.075	5.420	0.020	

a. ref = reference category for categorical variable; con = control group; tx = treatment group; HO = hatching order; CMI = cell-mediated immune response as measured by PHA (phytohaemagglutinin) skin test; CORT = corticosterone; CID = clutch initiation date; BS = brood size; IGR = instantaneous growth rate; TMT = tarsometatarsus length

Table 5.3. Models selected for factors affecting fate of Franklin's gulls within the first week of life, and from 7 days to fledging.

Variable[a]	*b*	SE	OR	OR 95% CL	*z*	*P*	*n*
Model 9 – Fate to 7 days (mortality = 1, survival = 0)							212
Constant	-6.675	1.961					
Tx (ref = con)	1.969	1.041	7.2	0.94 - 55.1	1.89	0.059	
HO2 (ref = HO1)	0.481	1.096	1.6	0.19 - 13.9	0.44	0.661	
HO3 (ref = HO1)	3.228	1.144	25.2	2.7 - 237.5	2.82	0.005	
Neonatal mass	-2.219	0.925	0.11	0.02 - 0.67	-2.40	0.016	
Model 10 – Fate after 7 days (mortality = 1, survival = 0)							176
Constant	-5.964	1.400					
Tx (ref = con)	0.982	0.865	2.7	0.49 - 14.5	1.14	0.256	
HO2 (ref = HO1)	1.389	1.209	4.0	0.38 - 42.9	1.15	0.251	
HO3 (ref = HO1)	3.464	1.241	31.9	2.8 - 363.7	2.79	0.005	
Mass at 2 weeks	-0.776	0.337	0.46	0.24 - 0.89	-2.31	0.021	
Neonatal CMI	-1.247	0.478	0.29	0.11 - 0.73	-2.61	0.009	

a. ref = reference category for categorical variable; con = control group; tx = treatment group; HO = hatching order; CORT = corticosterone; IGR = instantaneous growth rate; CMI = cell-mediated immune response as measured by PHA (phytohaemagglutinin) skin test; OR = odds ratio

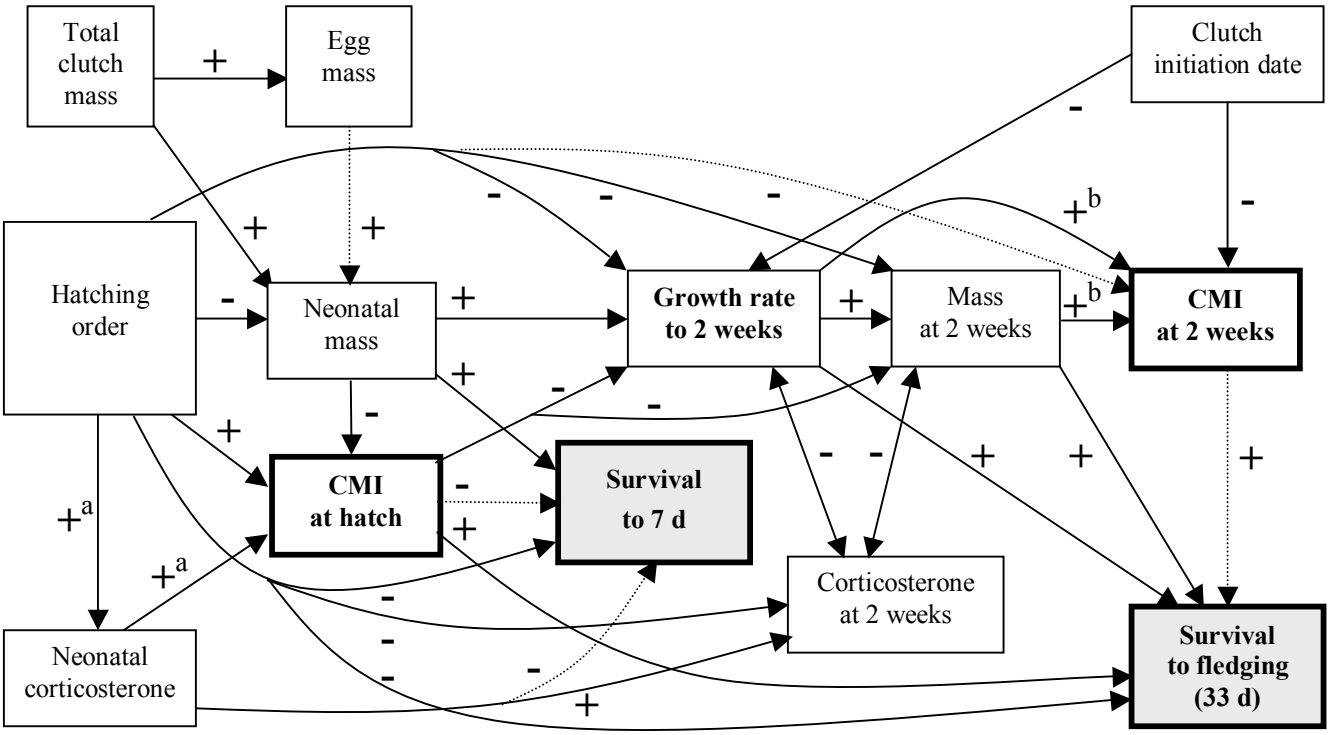

Figure 5.1. Observed relationships among hatching order, mass, CMI response, growth rate, and survival at two different stages in the development of Franklin's gull chicks, based on results obtained from multilevel models. Solid lines represent relationships significant at $P < 0.05$ in final models; dotted lines represent relationships significant at $P < 0.10$ when examined alone in models, or significant at $P < 0.05$ when examined alone in models, but weakened when in combination with variables in final models. [a]LO3-HO3 chicks had the highest corticosterone levels; only they experienced a positive correlation between corticosterone and CMI response. [b]HO3 chicks only.

6. GENERAL DISCUSSION

The concept of trade-offs is central to life history theory (Roff 2002), and is not novel; ecologists have been examining the role of trade-offs in life history evolution for decades. This concept is based on a presumption that investments in traits which directly or indirectly improve fitness will impose some sort of cost (e.g., energy, nutrients, proteins, time), and if there is a common currency among traits, they should trade-off with each other when the currency is limited. For instance, if some limited internal resource was not sufficient to cover all costs associated with maintenance and survival along with two life history traits, then a compromise between the competing processes must be reached; an increment of resources directed to one trait may cause a reduction of resources to the other trait (van Noordwijk & de Jong 1986; Zera & Harshman 2001). The importance of how 'parasitism' might shape host life history evolution has increasingly been examined over the last decade, and has recently branched to include the role of immune function as another competing life history trait, a topic that has received considerable attention in the avian literature in recent years. Despite the latter efforts, the concept of costs and trade-offs of life history traits has not been recognized widely as being relevant or important by traditional wildlife disease specialists, pathologists, or parasitologists involved with diagnosis of disease and identification of pathogens of wildlife.

6.1 To respond or not to respond?

The relationship between an infectious agent and its host is the result of a process of coevolution, in which changes in one influence changes in the other (Thompson 1994).

Infectious organisms keep evolving to evade the immune defenses of their hosts. Likewise, complex mechanisms of immune defense have evolved in response to infectious pathogens which may exert selection pressures on hosts by reducing survival or reproduction. Not responding to an agent can have severe fitness consequences by increasing the likelihood of disease and mortality, or by allowing sublethal effects such as reduced growth or reproduction. However, upregulation of the immune system in response to a disease agent also comes at a cost, the magnitude of which depends on the type of agent, type of response, and, as discussed below, the context in which the interaction occurs. Even responses to non-replicating, non-pathogenic antigens such as PHA and SRBC have been shown to increase metabolism (Ots *et al.* 2000; Martin *et al.* 2003), reduce growth rate (Fair *et al.* 1999; Chapter 5), or impair reproduction (Ilmonen *et al.* 2000; Råberg *et al.* 2000) in many species. Immune responses to actual infections (especially to viruses, bacteria, or protozoa) may be even more energetically or nutritionally costly because they often involve additional processes such as fever and anorexia. If an infection develops into disease despite active immune responses, costs rise even higher due in part to direct effects of the pathogen itself, as well as the host's ongoing responses involving cytokines or other mediators which can simultaneously increase metabolism and worsen cachexia, resulting in weight loss (Yuill 1987). Furthermore, an overzealous immune response may result in immunopathology, e.g., hypersensitivity reactions or immune-mediated diseases as has been described in humans and domestic animals. Because of the costs associated with upregulation of the immune system, along with potential fitness costs associated with not responding to an infectious organism, the immune response has been described as a double-edged sword (Hanssen *et al.* 2004); hence, a balance between the costs and benefits is likely optimal. Along with the arms race theory, selection for

optimal, rather than maximal, immune responses may partially explain why infectious organisms have not been eliminated by host immune systems (Zuk & Stoehr 2002).

6.2 The concept of priority rules

When competition for limited resources occurs among life history traits, priority rules may exist for 'deciding' which processes take precedence over others (Zera & Harshman 2001); however, it is also possible that priority rules shift depending upon the environment, season, and the individual's age, sex, stage of development, reproductive status, and condition. In other words, priority rules are not static (Zera & Harshman 2001), and are likely context-dependent. In some situations, immune function or the stress response may take precedence at the expense of reproduction; in other situations, activities related to reproduction may take precedence over immune function or maintenance costs. For instance, third-hatched Franklin's gull chicks in poor body condition should allocate resources primarily to maintenance and survival, at the expense of both growth and immune function if necessary. Priority rules for first-hatched chicks in good condition are not as critical, and resources may be directed to both growth and immune function which may or may not trade off with each other, depending upon resource availability and exposure to infectious agents. Animals in isolated environments may experience reduced levels of exposure to infectious pathogens; hence, it may be advantageous for immune function to be down-regulated in these species when in such environments. For instance, in some avian species breeding in Arctic environments, the stress response to adverse weather conditions may be down-regulated to prevent brood failure caused by interrupted incubation, as opportunities to renest during the very short breeding season may not be available (Wingfield *et al.* 1995).

6.3 The birds and the bees: Context-dependent costs of immune responsiveness

Immune responsiveness against a potential pathogen is usually considered beneficial because it should offset the costs of infection and disease, and reduce the risk of mortality. However, depending upon the context, the cost of immune activation has the potential to outweigh the benefits, leading to reduced survival. In such situations, priority rules involving immune function may shift towards down-playing the importance of the immune system, as is discussed below.

The most convincing evidence for context-dependent costs of immune function exists in the invertebrate literature. Bumble bees (*Bombus terrestris*) that were starved and immune-challenged experienced higher mortality compared to bees that were starved and not challenged, or bees that were challenged and fed *ad libitum* (Moret & Schmid-Hempel 2000). Immune system activation in the *ad libitum*-fed bees had no effect on survival. In free-ranging vertebrates, convincing evidence for context-dependent costs of immune responses comes from a study examining immune function in female common eiders (*Somateria mollissima*), which fast during egg-laying and incubation, losing about 40% of their body weight during the reproductive period (Hanssen *et al.* 2004). Females that responded to SRBC vaccinations administered at the beginning of the incubation period had markedly reduced return rates (27%) the following year compared to females that did not respond (72%). Interestingly, return rates were even lower for females that responded to both SRBC and diphtheria toxoid than for females only responding to SRBC. Hence, upregulation of the immune system in response to non-pathogenic vaccinations or antigens during a stressful period (food-deprivation) appeared to have severe negative consequences for long-term survival of eiders, and short-term survival of bees. In the eiders, 21% of the immunized birds

did not respond to any of the three administered vaccines (SRBC, diphtheria toxoid, and tetanus), and it was suggested that immunosuppression during this time period is an evolutionary adaptive response (Hanssen *et al.* 2004). This interpretation is plausible because such a marked reduction in return rate may exert strong selection pressure for the avoidance of costly immune responses during this predictable period of severe resource limitation, which also is a period during which the probability of exposure to infectious agents may be reduced due to the immobility and fasting behaviour of the female. Hence, it is possible that priority rules within the non-responding females changed during the incubation period, such that the costs of somatic maintenance and reproduction took precedence over the costs of immune function.

6.4 Mechanisms of immune function-life history trade-offs

There is a rapidly growing body of evidence demonstrating functional trade-offs between immune function and various life history traits. In the majority of studies, the physiological mechanisms underlying these trade-offs are unknown, and assumed to be based upon the differential allocation of limited internal stores of energy, nutrients, proteins, or other resources. In the eider and bee studies, the functional trade-offs observed between immune function and survival were likely resource-dependent, as severe consequences were observed only under conditions of food-deprivation. However, it is unknown whether the trade-offs were condition-dependent because neither of the two studies explored the role of condition for survival.[1] Nonetheless, to my knowledge, these are currently the only two studies that have demonstrated the resource-dependence of a trade-off. Evidence for the condition-dependence

[1] Mass or quality of individual was examined in the eider study, and was not associated with whether or not female eiders responded to SRBC, nor the magnitude of responses. Furthermore, females that responded to SRBC did not experience reduced mass, nor increased nest desertion in the short term. The effect of mass or quality on return rate was not examined or presented.

of resource-based trade-offs is still lacking. My study may be unique in providing evidence for the roles of both condition (mass) and social status (hatching order) in affecting or mediating trade-offs among CMI responses, growth, and survival. Despite being an observational study, the significant associations observed identify plausible mechanisms underlying the relationships or trade-offs between immune function and other life history traits. These mechanisms should be explored or tested further with manipulative studies, which may help further reveal the complex relationships among energy metabolism, endocrine control mechanisms, and the immune system. In general, further examination of the role of condition for trade-offs assumed to be resource driven, is essential. Are trade-offs between immune function and survival during periods of food-deprivation in bumble bees and female common eiders more obvious in individuals in poor condition compared to those in good condition? Are resource-based trade-offs among traits masked in some situations, and magnified in other situations, depending upon condition, stage of development, reproductive status, or food availablity (i.e., the context)? The use of manipulative studies may help shed light on these questions. One example could include manipulating body condition of hatchlings, and subsequently examining the effect of condition on the magnitude or existence of trade-offs between immune responsiveness and growth rate. Another might include comparing the effect of increasing the costs of immune responsiveness on the existence or magnitude of trade-offs. The cost of immune response may be increased by administering a compound that induces fever (e.g., LPS), or by supplementing the diet with methionine which enhances T-cell immunity, resulting in more robust CMI responses to antigens such as PHA (Soler *et al.* 2002; Brommer 2004). Also of value would be to determine the effect of shifting priority rules on trade-offs. For instance, would the relationship between immune function

and return rate differ among female eiders vaccinated at different times around the breeding period, e.g., before laying, during early incubation, during late incubation, or during brood care? How different would trade-offs be during migration, or on their wintering grounds? As is the breeding period, migration is a potentially stressful and costly process; hence, it may be beneficial to suppress immune responses to preserve energy or nutrients for flight. However, in contrast to the fasting and immobile incubating eiders, migrating birds may be at increased risk to exposure of infectious pathogens, so the costs of not responding to an agent may be higher within this context. Untangling the complexity of mechanisms underlying life history trade-offs and their priority rules may help us understand how immune responses (Zera & Harshman 2001) as well as the regulation of immune responses to infectious organisms (Wikelski & Ricklefs 2001) have evolved.

6.5 Future directions: Bridging gaps among disciplines

As mentioned at the beginning of this discussion, the idea of costs and trade-offs among life history traits involving immune function has not spilled over into the realm of traditional wildlife disease specialists, pathologists, and classical parasitologists, despite being an intensively studied topic in recent years. An ecologist looking into the wildlife pathologist's world, might suggest that exploring the concepts of life histories, costs, and trade-offs from a wildlife disease perspective may help improve our understanding of the mechanisms and impacts of parasitism, infection, and disease. When investigating a disease outbreak in a free-ranging population, or investigating an emerging infectious disease entering a new geographical area, or characterizing a new parasite within a host or an old parasite within a new host or geographical area, it is important to take into consideration the various factors related to the life history of the host species – including context-dependent trade-offs

potentially operating within individuals – that might predispose individuals to infection and disease. A pathologist looking into the ecologist's world might find it exciting that ecologists are recognizing that 'parasites' or 'infectious agents' may impose costs on their hosts, either directly or indirectly via costly upregulation of the immune system, thereby potentially shaping the evolution of host life history traits. However, valuable information can be obtained by incorporating the use of necropsy and other diagnostic techniques in life history trade-off studies to determine proximate causes of mortality, and to rule out infectious agents or toxins rather than assuming that an animal died of starvation or predation. Our knowledge of both disease mechanisms and life history theory would benefit greatly if we could tie together the proximate causes of mortality with specific life history traits or trade-offs occuring within individuals.

6.6 Synopsis and concluding remarks

The research comprising the body of this thesis examined aspects of two seemingly unrelated wildlife mortality events, namely, mortality caused by botulism in waterfowl, and mortality associated with hatching asynchrony in prefledgling Franklin's gulls. The sequence of discoveries characterizing the evolution of this thesis was a series of logical steps that connected the two mortality events. This study began with the exploration of factors involved in the initiation of avian botulism outbreaks. More specifically, the role of Franklin's gull mortality was examined as a potential primary source of carcass substrate required to precipitate outbreaks. From 1999 to 2001, hatch-year Franklin's gull carcasses were the predominant source of toxin-laden maggots found prior to outbreaks of avian botulism in waterfowl. Peak carcass density of gulls occurred 1-2 weeks prior to the onset of botulism outbreaks in waterfowl in each year. Hatch-year gull carcasses were suitable substrate for

production of type C toxin by *C. botulinum*, and were the predominant source of substrate for both toxin production and maggot development on transects, beginning at least 10-11 days prior to the first detected cases of botulism in waterfowl. High density of toxic material from hatch-year Franklin's gull carcasses prior to the onset of botulism coincided with high densities of susceptible birds; hence, gull mortality had the potential to be a major initiating factor for botulism outbreaks at Eyebrow Lake. Examination of the causes of gull mortality revealed an assortment of conditions or diseases associated with stress, starvation, and immunosuppression, and most of the mortality occurred among third-hatched chicks. The role of hatching asynchrony was further investigated, and it was determined that hatching order, independent of laying order, significantly affected survival to fledging, whereas laying order had no observable effect. More detailed investigations revealed the complexity of relationships among condition, growth, immune function, corticosterone, and survival, and how these relationships may be affected by hatching asynchrony and stage of development. As discussed in the last section, this study also provided support for the condition-dependence of trade-offs, and that trade-offs may change with stage of development, being more prominent during periods of rapid growth.

It is intriguing how a mortality event in one species (Franklin's gulls) may influence or help trigger a completely different mortality event in other species (waterfowl and other susceptible avian species). The increased risk for botulism is a coincidental effect of the normal, predictable prefledgling gull mortality associated with hatching asynchrony, and does not benefit the gulls directly (although there may be a benefit to the bacterium and phage). In the absence of gull mortality, it is possible that botulism outbreaks might still occur at Eyebrow Lake (as happens on many wetlands without nesting gulls) if other sources of

substrate were available (e.g., from nestlings of other marsh-nesting species, from sudden mortality events caused by hailstorms, lightning, etc., or from invertebrate carcasses). However, the presence of a large colony of gulls at Eyebrow Lake is a risk factor that must be considered if botulism were to be managed. Management implications were discussed in Chapter 3, however, some may speculate that possible management strategies for preventing botulism outbreaks on lakes similar to Eyebrow Lake could include preventing the nesting of Franklin's gulls (e.g., by changing water levels), reducing nesting densities, destroying nests, or reducing hatch rates of chicks per nest (e.g., by oiling or addling eggs). The decision is partially one involving a value judgement based on choosing one species over another. If the decision is to manage the gull colony in some fashion, the gull population should be monitored for negative impacts, and strategies must be evaluated for their effectiveness in reducing mortality caused by botulism, prior to being implemented in routine management plans.

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APPENDIX A. LIST OF EQUATIONS[1] EMPLOYED IN CHAPTER 3

Equation A1. $N = \frac{nA}{a}$, where N is the estimated total number of objects (nests or carcasses) in the gull colony, n is the number of objects counted on strip transects, A is the total colony area, and a is the strip transect area.

Equation A2. $a = 2Lw$, where a is the area surveyed, L is total transect length on the colony, and w is the strip half-width (i.e., 5 m).

Equation A3. $\hat{D} = \frac{n}{2Lw}$, where $\hat{D}$ is the estimated density of objects (nests or carcasses) in the colony using the strip transect method

Equation A4. $\hat{D} = \frac{n}{a \cdot \hat{P}_a}$, where $\hat{P}_a$ = probability of nest detection in area a.

Equation A5. $\hat{var}_{\hat{D}} = \hat{D}^2 \cdot \left\{ \frac{\hat{var}_n}{n^2} + \frac{\hat{var}_{\hat{f}(0)}}{\left[\hat{f}(0)\right]^2} \right\}$, where $f(0)$ is the value of the probability density function of perpendicular distances, evaluated at 0 distance; the value is equivalent to the proportion of detected objects observed at 0 distance.

[1] from Burnham *et al.* (1980) and Buckland *et al.* (2001).

Equation A6. $\hat{var}_n = \dfrac{L\sum_{i=1}^{k} l_i (n_i / l_i - n/L)^2}{k-1}$

Equation A7. 95% CL, lower = $\hat{D}/C$ and upper = $\hat{D} \cdot C$, where C is a constant as estimated below

Equation A8. $C = \exp\left(z_\alpha \sqrt{\hat{var}_{\ln \hat{D}}}\right)$, where $z_\alpha = z_{0.025} = 1.96$

Equation A9. $\hat{var}_{\ln \hat{D}} = \ln\left[1 + \dfrac{\hat{var}_{\hat{D}}}{\hat{D}^2}\right]$

APPENDIX B. MODELS CREATED IN DISTANCE 4.0

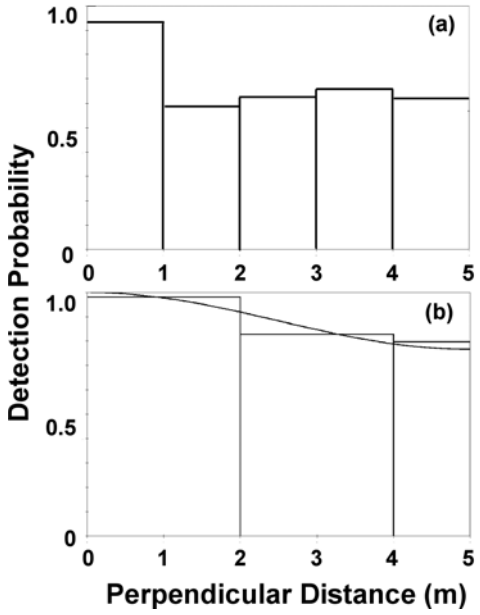


Figure B1. Distance data of Franklin's gull nest survey in 2001. Histogram in (a) shows distance data grouped in 1 m intervals to 5 m. Data grouped in 2 m intervals (b) provided a gradual reduction in detection from centreline, and removed the spike observed at 0-1 m in (a). Curve in (b) represents the detection function estimated with a uniform + cosine model which provided the best fit for data grouped in this way (χ^2 = 0.28; P = 0.6). According to this model, $\hat{P}_{a_{nest}}$ in 2001 was 0.88.

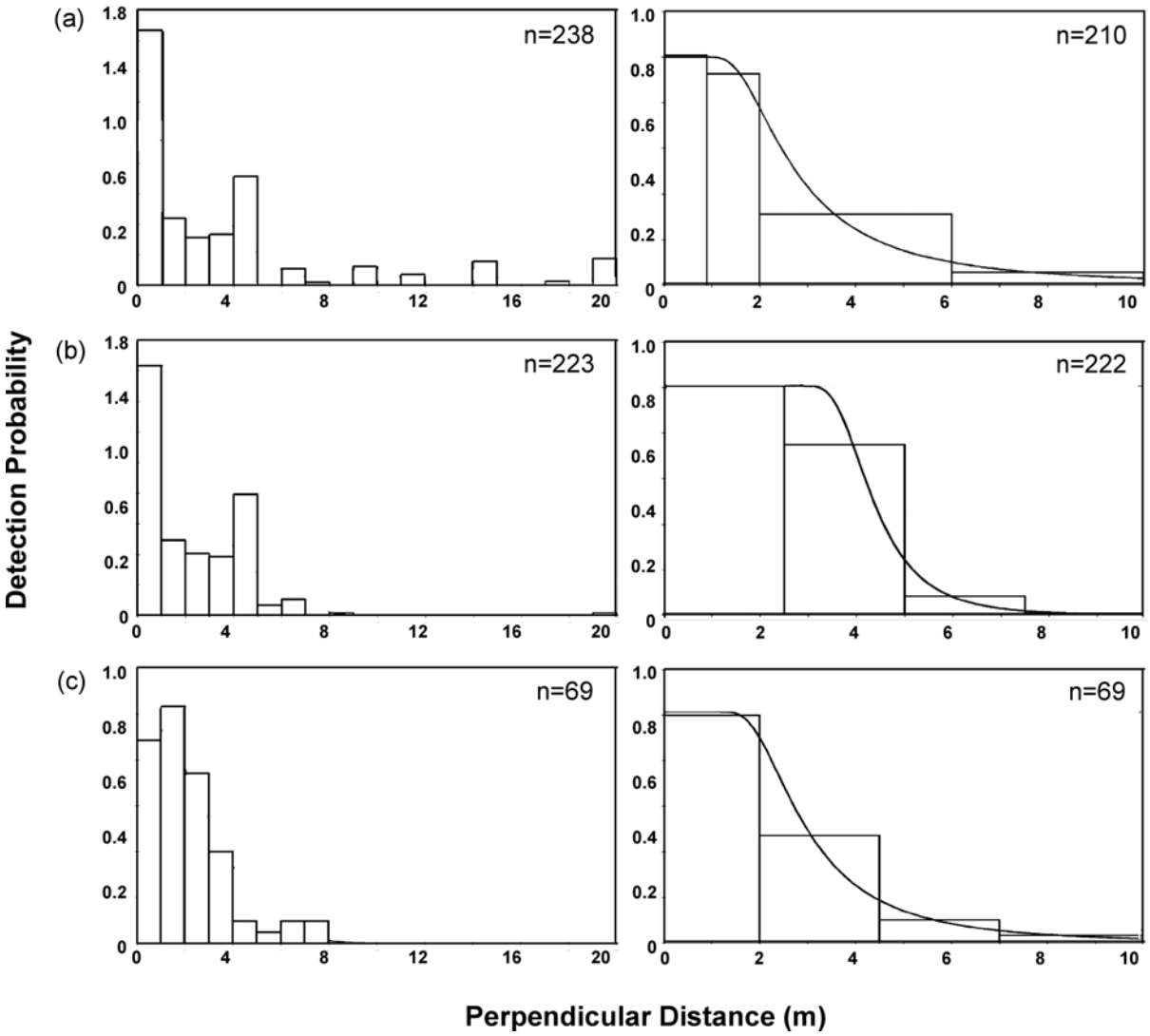


Figure B2. Best models for cumulative density of juvenile Franklins's gull carcasses using pooled data from weekly visits to transects, from late May to late July in 1999 (a), 2000 (b), and 2001 (c). Distance data were initially examined with data grouped in 1 m intervals (left column). A spike at the 0 - 1 m interval and heaping at 5 m were observed in both 1999 and 2000. Violation of the assumption of complete censusing of the centreline ($g(0) < 1$) occurred in 2001. Grouping of the distance data was performed to reduce these effects (right column). Data were truncated to 10 m in 1999 and 2000; no truncation was performed for 2001 data. For each year, the best model for grouped data was the hazard rate + cosine model, as illustrated by the fit of detection function curves on histograms ($\chi 2 = 0.0013$, P = 0.97; $\chi 2 = 0.0008$, P = 0.98; and $\chi 2 = 0.0053$, P = 0.94, respectively).

APPENDIX C. MAPS OF WEEKLY MORTALITY ON EYEBROW LAKE, 1999-2001

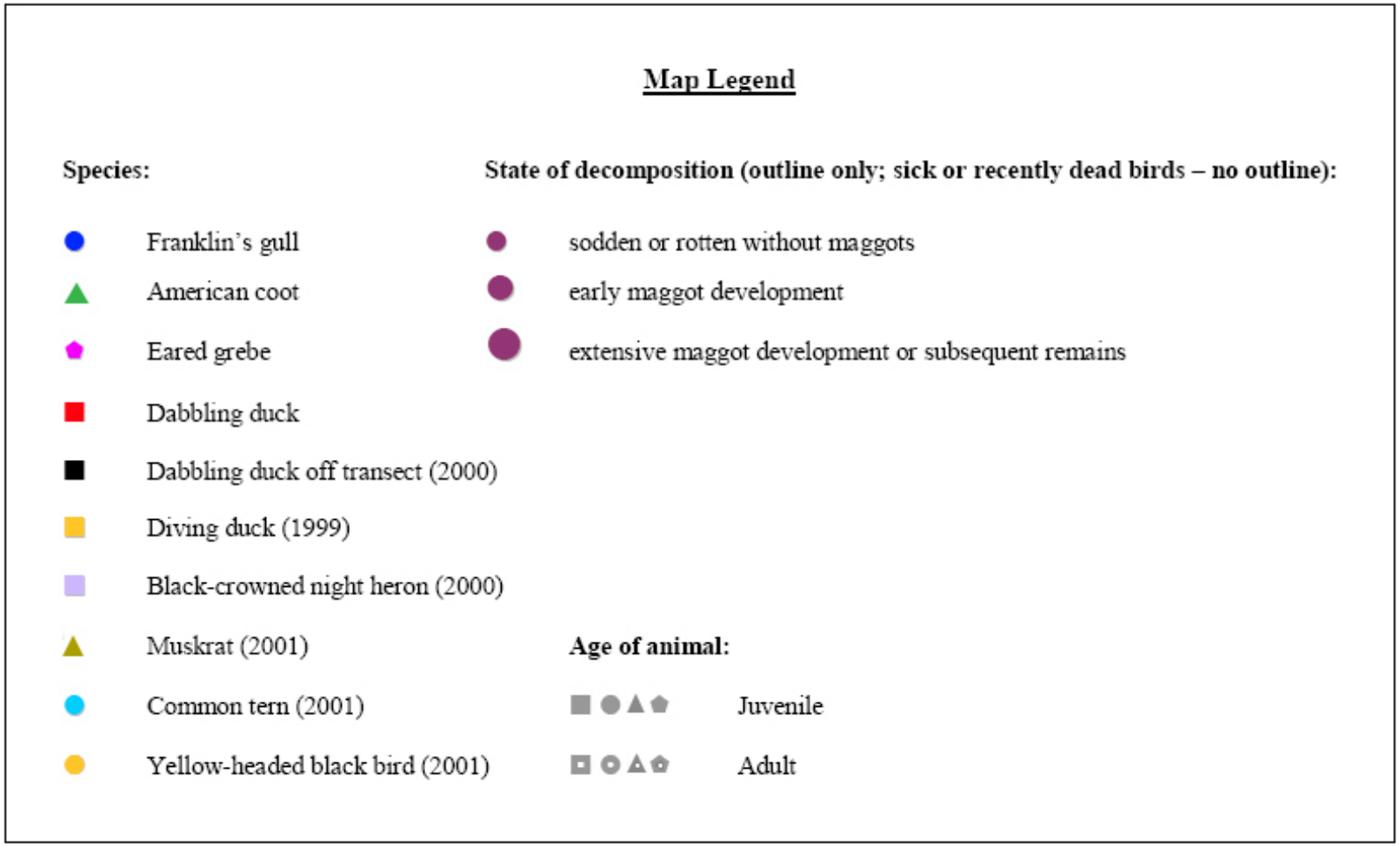
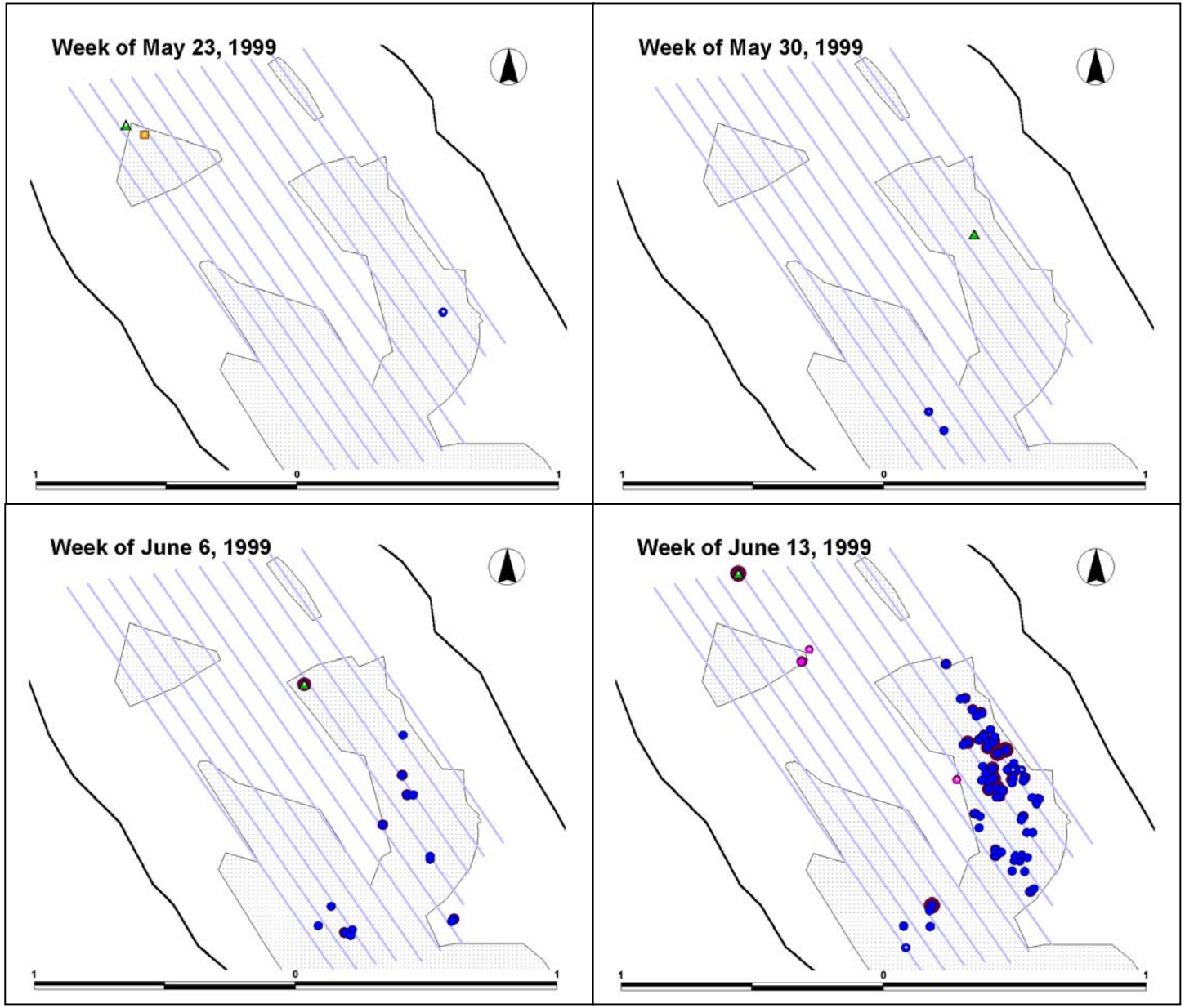
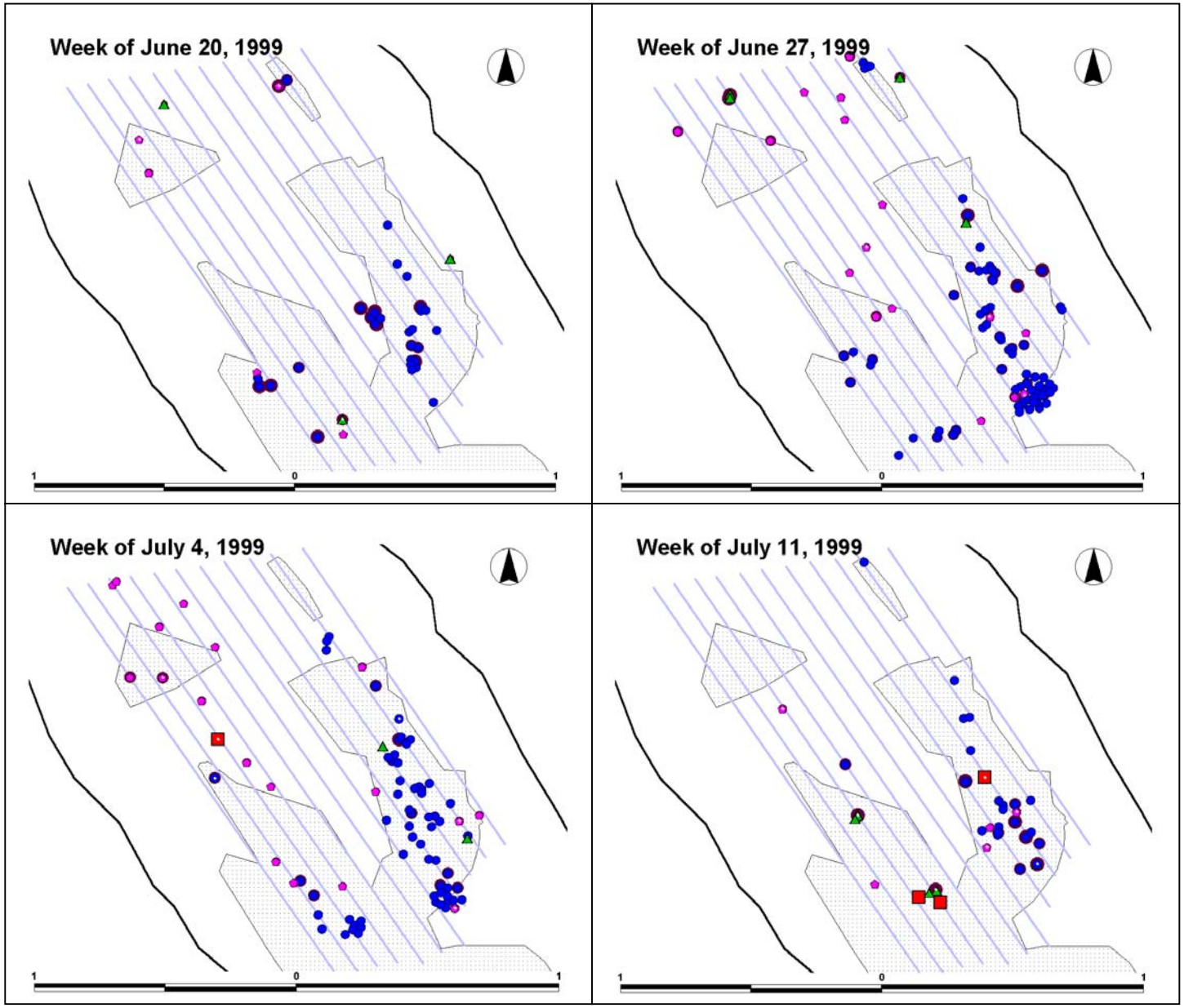


Figure C1. Weekly mortality on transects at Eyebrow Lake in 1999 - 2001. Shaded area represents gull colony, and parallel lines indicate location of transects. For point of reference, see Figure 3.1 for maps illustrating the entire lake and gull colony for each year.

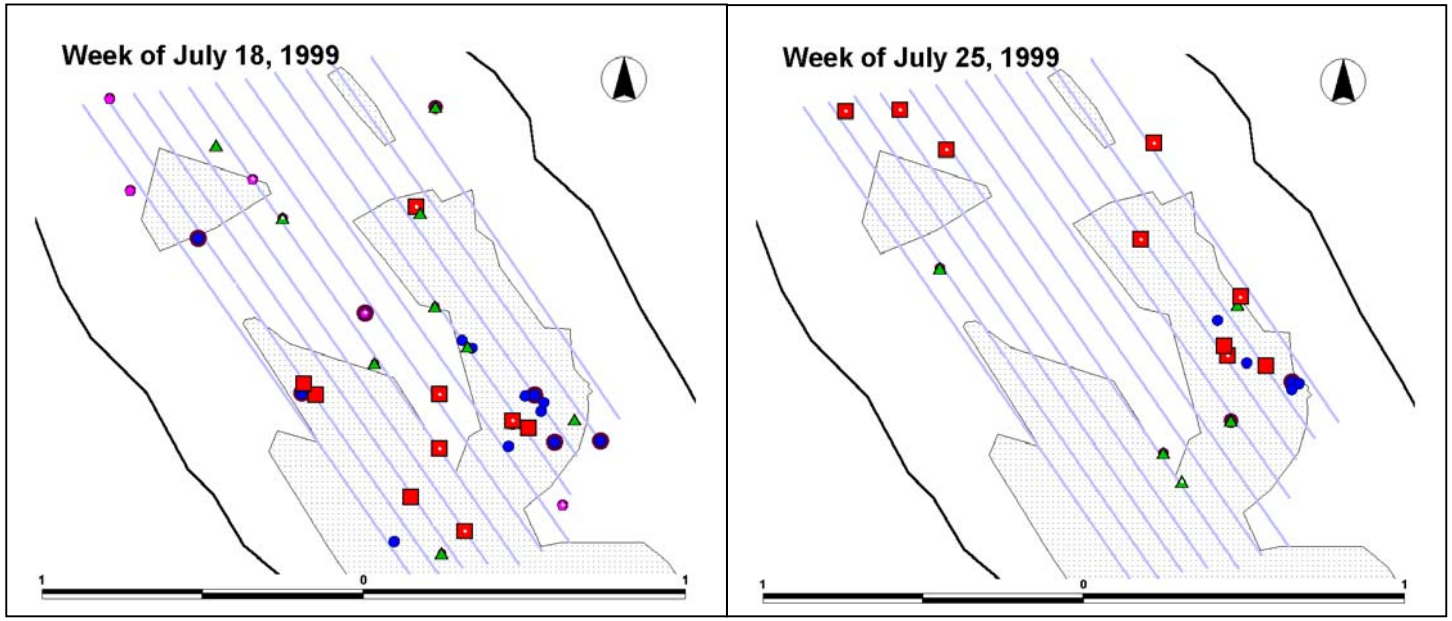
1999


Week of May 23, 1999
Week of May 30, 1999
Week of June 6, 1999
Week of June 13, 1999

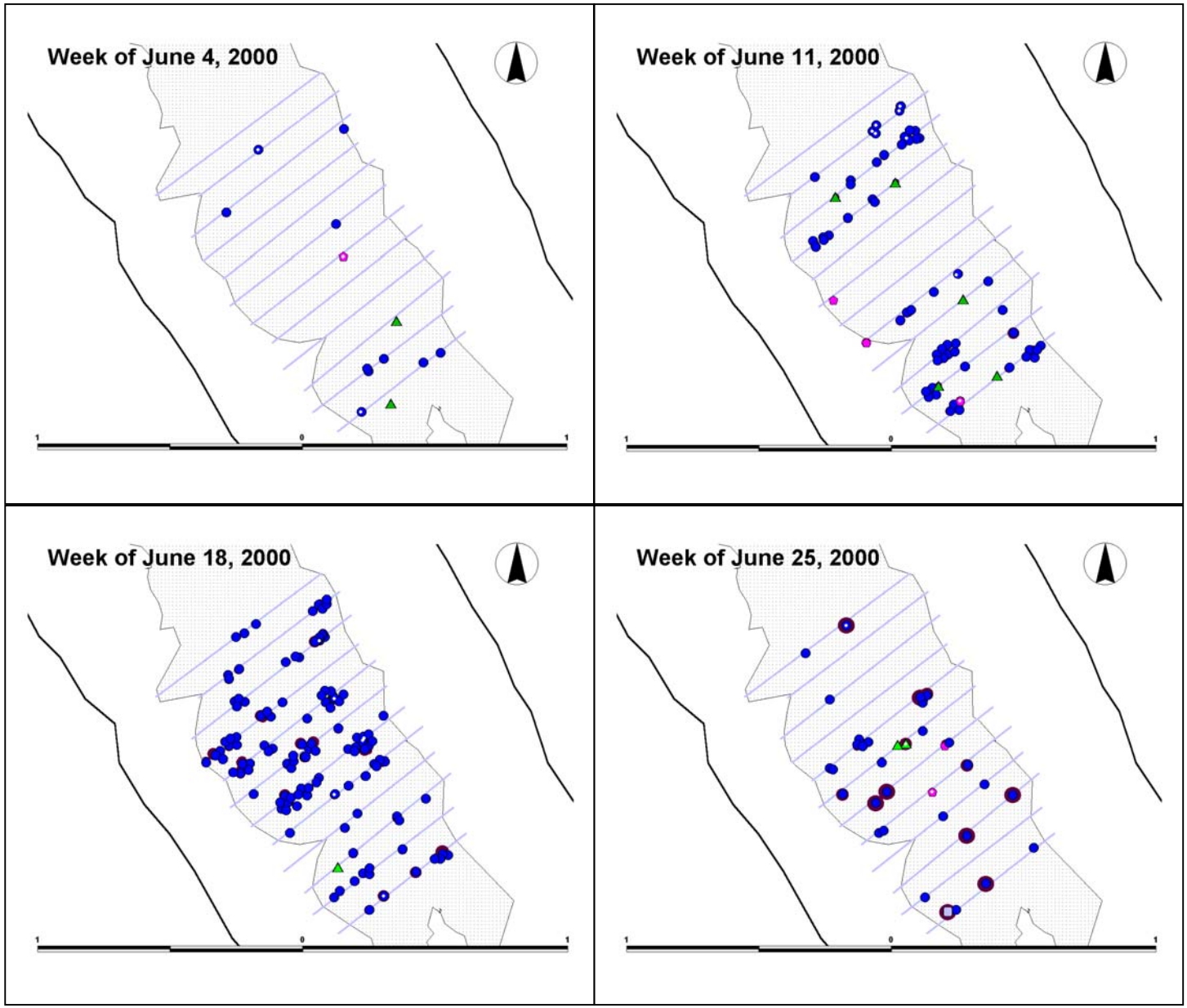
1999 cont'd


Week of June 20, 1999
Week of June 27, 1999
Week of July 4, 1999
Week of July 11, 1999

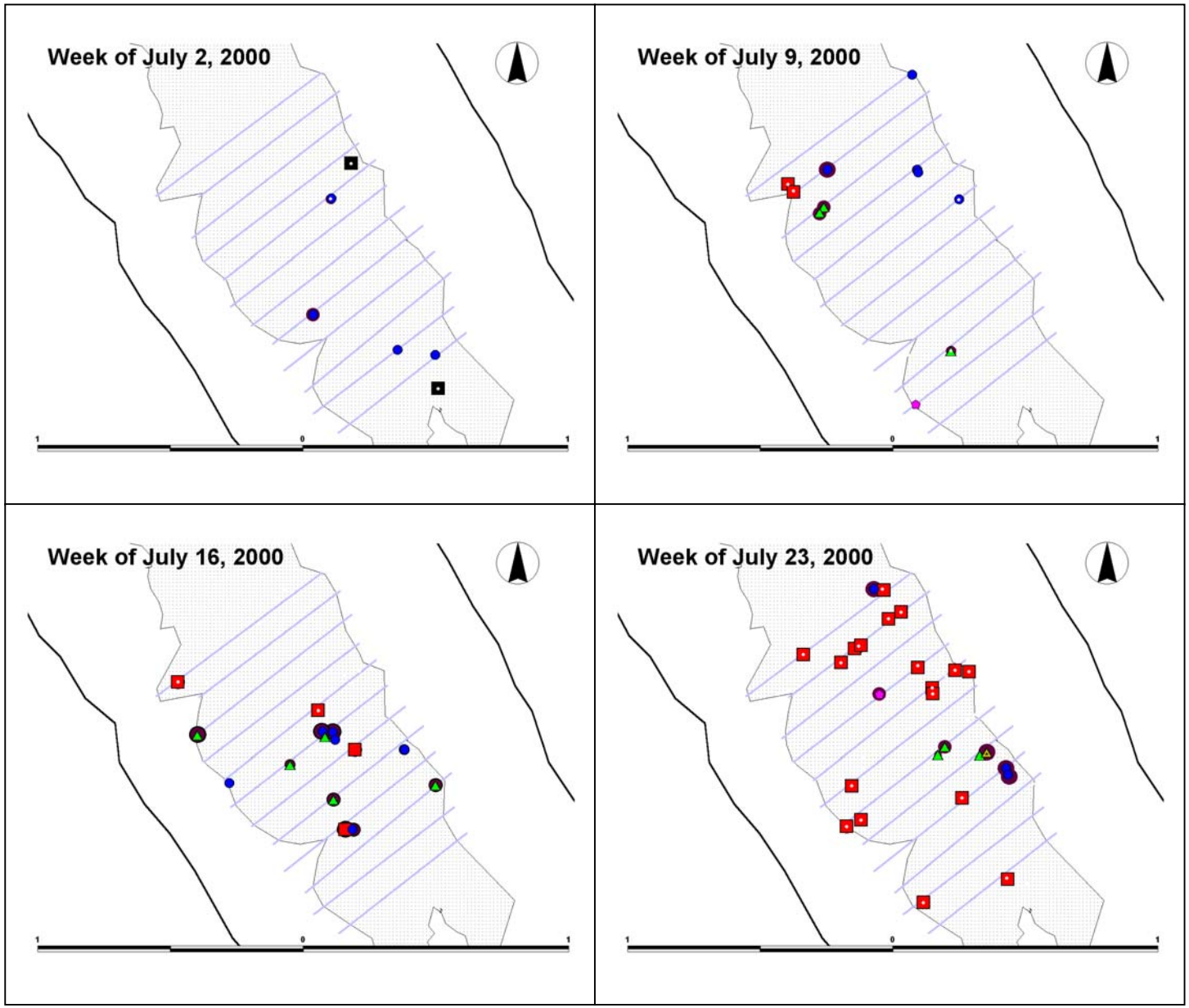
1999 cont'd



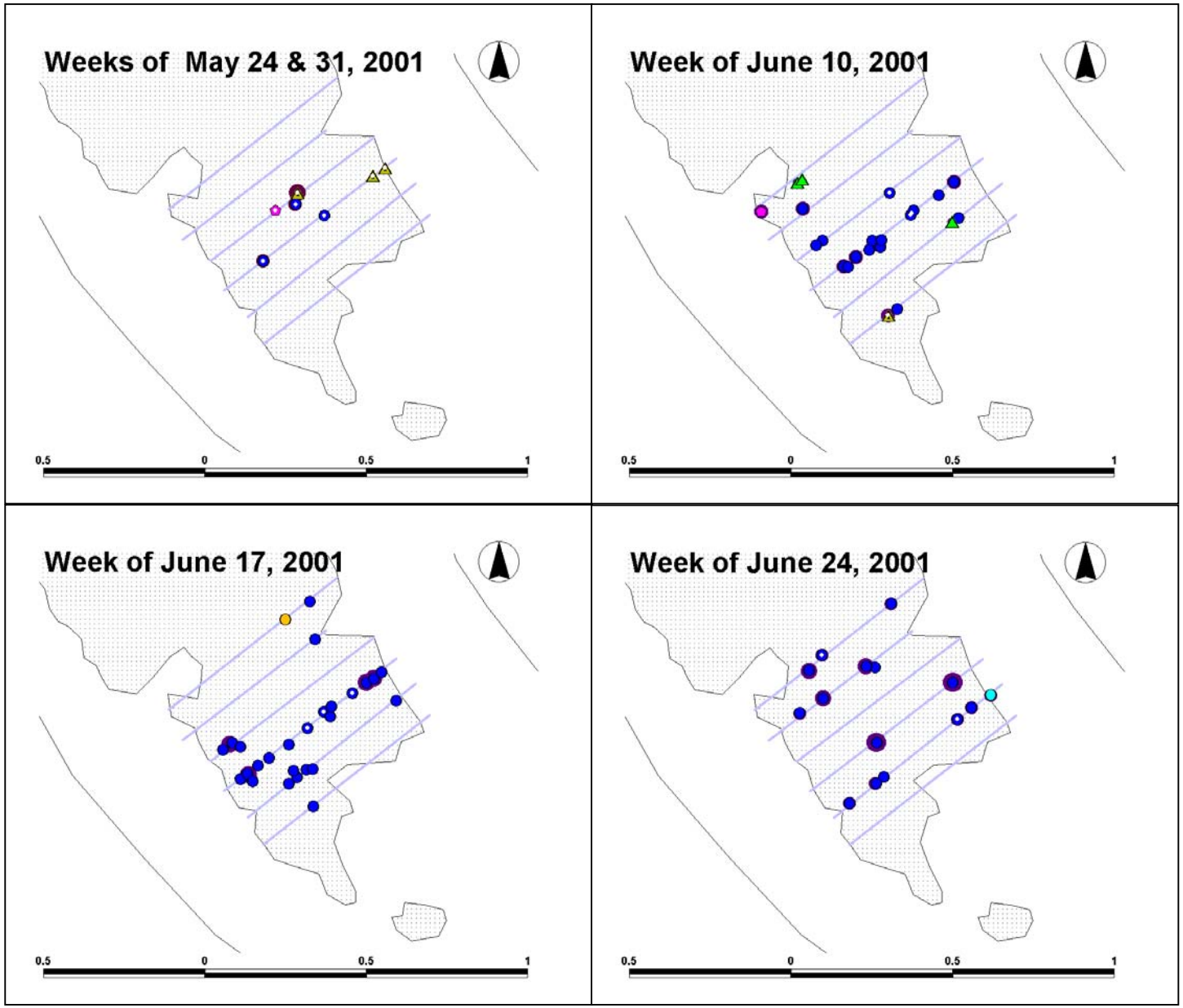
2000


Week of June 4, 2000
Week of June 11, 2000
Week of June 18, 2000
Week of June 25, 2000

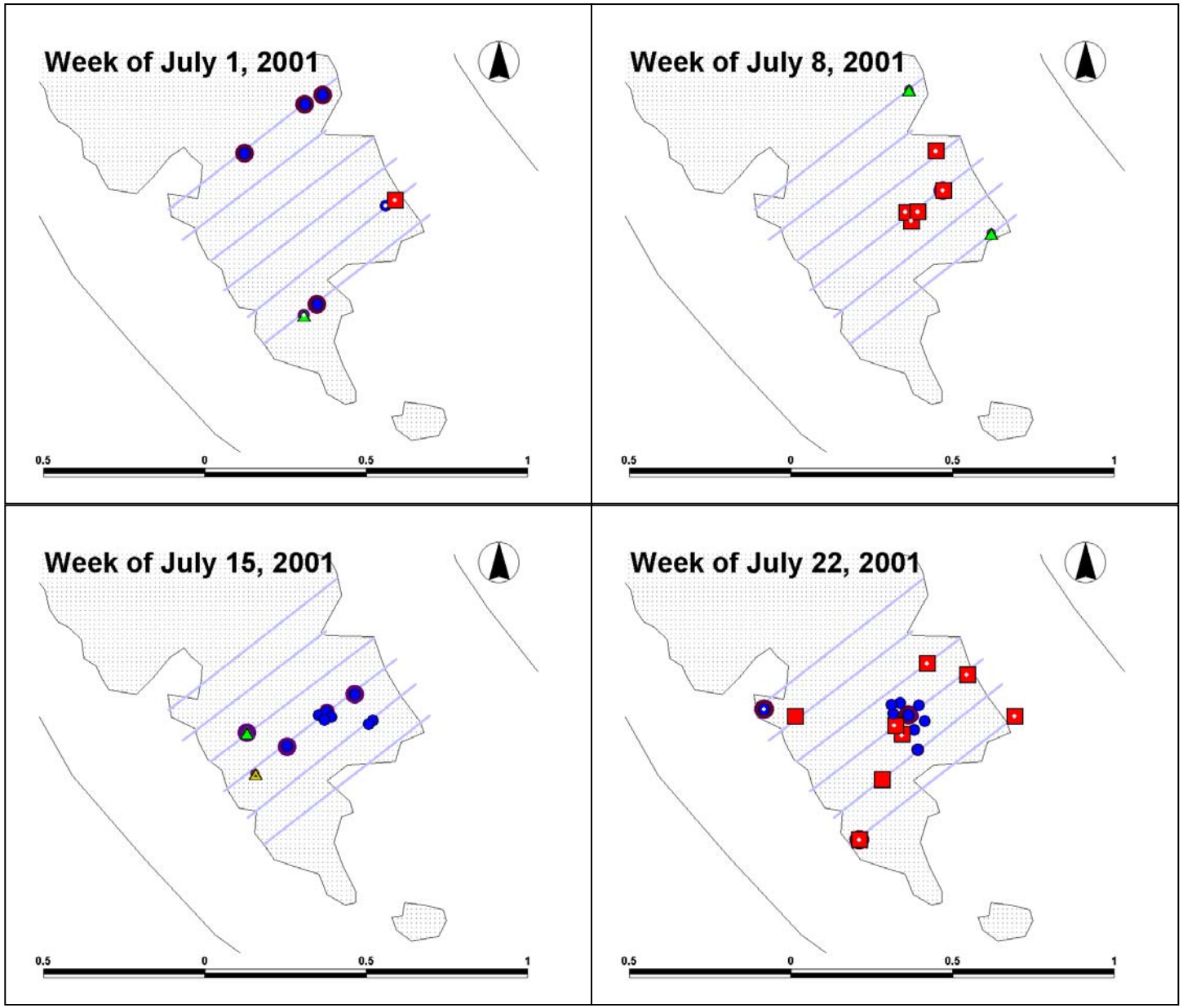
2000 cont'd


Week of July 2, 2000
Week of July 9, 2000
Week of July 16, 2000
Week of July 23, 2000

2001


Weeks of May 24 & 31, 2001
Week of June 10, 2001
Week of June 17, 2001
Week of June 24, 2001
0.5
0
0.5
1

2001 cont'd


Week of July 1, 2001
0.5
0
0.5
1
Week of July 8, 2001
0.5
0
0.5
1
Week of July 15, 2001
0.5
0
0.5
1
Week of July 22, 2001
0.5
0
0.5
1

APPENDIX D. PROXIMATE CAUSES OF FRANKLIN'S GULL MORTALITY

Detailed gross and histopathological analyses were performed on dead juvenile Franklin's gulls collected during transect surveys in 1999, 2000, and 2001 (Chapter 3), and during the cross-fostering study in 2001 (Chapter 4 and 5). Tissues routinely examined by histopathology included lung, heart, liver, kidney, spleen, thymus, bursa of Fabricius, cecal tonsils, all regions of the gastrointestinal tract, pancreas, adrenal gland, brain, peripheral nerve, thyroid gland, parathyroid gland, gonad, and pectoral muscle. Sections of other tissues (e.g., skin, bone, air sac, etc.) were examined microscopically if lesions were observed on gross examination. Samples of liver, lung, kidney, and bone marrow were frozen at -20°C until processed for routine bacteriological culture plus culture for *Salmonella* spp. Electron microscopy was employed where appropriate to identify viral inclusions detected histopathologically.

Table D1 is a summary of the primary causes of death in gulls for each year of the study. Carcass mass was employed as a rough estimate of chick age, based on the carcass mass of chicks of known age from the cross-fostering study performed in 2001. The 95% confidence limits for carcass mass were 20.7 – 25.6 g for chicks that died within the first week (n = 27), 40.3 – 117.8 g for chick that died during the second week (n = 8), and 113.4 – 187.8 g for chicks older than two weeks (n = 11). Using the midpoint between upper and lower confidence limits of the first and second week, respectively, a cut-off mass of 33 g was employed to classify chicks into the two age categories displayed in Tables D1 and D3. Contributing factors associated with each primary cause of mortality for each year are

displayed in Table D2, and common diagnoses are presented in Table D3. Of the cases in which disease was the primary cause of mortality, pneumonia, inflammation involving the heart, bacteremia, or some combination of these were the most common diagnoses each year.

Although this section was not meant to include an interpretation of the data presented, a few interesting observations should be highlighted. Disease as the immediate cause of death was diagnosed uncommonly in chicks less than one week old, which more commonly drowned or succumbed to trauma associated with sibling rivalry, conspecific aggression, or predation, often in association with starvation. Chicks older than one week (from transect studies) that died primarily of disease were generally emaciated, and often had moderate to marked lymphoid depletion of the bursa of Fabricius. In 1999, there appeared to be a higher proportion of cases with disease as the primary cause of mortality compared to 2000 and 2001. This was primarily due to the large number of cases of *Staphylococcus aureus* septicemia. The most likely modes of infection for *S. aureus* were secondary to trauma of the head associated with sibling or conspecific aggression, or through the foot web secondary to larval schistosome penetration which was not observed in 2000 and 2001. The observation that diseases and disease patterns vary from year to year illustrates the importance of long-term studies involving wildlife disease mortality.

Table D1. Summary of the primary causes of death in juvenile Franklin's gulls at Eyebrow Lake. Gulls were collected during transect and island surveys in 1999, 2000, and 2001, and the cross-fostering study (fenced chicks) in 2001.

Primary cause of death[a]	Number of cases											
	1999			2000			2001					
							Survey			Fenced chicks[b]		
	≤ 7 d	> 7 d	Total	≤ 7 d	> 7 d	Total	≤ 7 d	> 7 d	Total	≤ 7 d	> 7 d	Total
Starvation	0	0	0	0	4	4 (12.9%)	1	0	1 (3.3%)	7	5	12 (26.1%)
Disease	0	14	14 (26.4%)	0	2	2 (6.5%)	0	5	5 (16.6%)	4	2	6 (13.0%)
Trauma	4	3	7 (13.2%)	0	3	3 (9.7%)	0	1	1 (3.3%)	7	5	12 (26.1%)
Predation	0	1	1 (1.9%)	1	7	8 (25.8%)	1	9	10 (33.3%)	4	6	10 (21.7%)
Drowning	3	2	5 (9.4%)	0	1	1 (3.2%)	3	4	7 (23.3%)	5	1	6 (13.0%)
Euthanised	0	26	26 (49.1%)	0	13	13 (41.9%)	0	6	6 (20.0)	0	0	0
Total	7	46	53	1	30	31	5	25	30	27	19	46

[a] The primary causes of death are defined as:

Starvation: diagnosed based on emaciation of carcass, i.e., complete lack of fat stores, and moderate to marked reduction of pectoral muscle mass.

Disease: life-threatening disease affecting major organs; caused by infectious organisms (e.g., bacteria, viruses) or, in a few cases, by botulinum toxin.

Trauma: acute trauma caused by sibling or conspecific aggression; hemorrhage or lesions restricted to head region.

Predation: more severe traumatic lesions caused by predator, as evidenced by hemorrhages or bruising on head, neck, back, with occasional fractured bones, decapitation, or puncture marks.

Drowning: as evidenced grossly by red, congested lungs and congested atria, and histologically by edematous, fluid-filled airways, often containing refractile foreign material, together with congestion and/or hemorrhage.

Euthanised: chicks that were moribund or showed signs of illness were euthanised by cervical dislocation.

[b]Fenced chicks were observed until fledging; hence, causes of death in fledged juveniles were unknown. The majority of individuals from transects euthanised in 1999, 2000, and 2001 were fledged juveniles.

Table D2. Contributing factors associated with the primary causes of death in juvenile Franklin's gulls in 1999, 2000, and 2001. Sample sizes are displayed along the top line of each section, but are reported as denominators where different.

	Primary cause of death						
Contributing factors	Starvation	Disease	Trauma	Predation	Drowning	Euthanised	Total
1999 – Chicks from surveys	***n* = 0**	***n* = 14**	***n* = 7**	***n* = 1**	***n* = 5**	***n* = 26**	***n* = 53**
Emaciation[a]	-	9	7	0	3/3	16	28/48
Disease/Infection	-	14	4	1	2	24	45
Life-threatening disease[b]	-	14	3	0	2	20	39
Evidence of trauma history[c]	-	11/13	2	1	2	8	24/52
Parasitism[d]	-	5	1	1	2	22	31
Tissue pallor or anemia[e]	-	1	4	0	1	10	16
Lymphoid depletion[f]	-	7/9	1/6	0	3/5	4/19	15/40
2000 – Chicks from surveys	***n* = 4**	***n* = 2**	***n* = 3**	***n* = 8**	***n* = 1**	***n* = 13**	***n* = 31**
Emaciation	4	2	3	4/7	0	6	19/30
Disease/Infection	3	2	3	5	1	12	26
Life-threatening disease	0	2	1	3	0	11	17
Evidence of trauma history	2	1	3	5	0	3	14
Parasitism	0	0	0	1	1	3	5
Tissue pallor or anemia	0	0	2	0	0	5	7
Lymphoid depletion	3	0	0	3	0	2	8
2001 – Chicks from surveys	***n* = 1**	***n* = 5**	***n* = 1**	***n* = 10**	***n* = 7**	***n* = 6**	***n* = 30**
Emaciation	1	5	1	8	2/6	2/5	19/28
Disease/Infection	0	5	1	3	4	6	19
Life-threatening disease	0	5	0	1	1	2	9
Evidence of trauma history	0	4	1	6	2	3	16
Parasitism	0	3	0	2	2	5	12
Tissue pallor or anemia	0	4	1	3	1	1	10
Lymphoid depletion	0	3	1	4/9	4	0	12/29
2001 – Fenced chicks	***n* = 12**	***n* = 6**	***n* = 12**	***n* = 10**	***n* = 6**	***n* = 0**	***n* = 46**
Emaciation	12	2	9	1	3	-	27
Disease/Infection	5	6	6	6	2	-	25
Life-threatening disease	0	6	1	4	1	-	12
Evidence of trauma history	2	1	3	4	2	-	12
Parasitism	1	0	1	0	0	-	2
Tissue pallor or anemia	7	2	1	1	3	-	14
Lymphoid depletion	2	5	3	1	3	-	14

[a]complete lack of fat stores, and moderate to marked reduction of pectoral muscle mass.
[b]life-threatening diseases involved moderate to severe inflammation of at least one major organ (e.g., lung, heart, brain), often in conjunction with bacteremia. Confirmed botulism was also included in this category. Non-life-threatening diseases involved mild inflammation of one or more organs, plus or minus bacteremia.
[c]mostly chicks with dermatitis with or without myositis of the head, associated with sibling or conspecific aggression.
[d]based upon gross or histological observation of moderate to marked levels of gastrointestinal helminths (e.g., cestodes, trematodes, nematodes, acanthocephalans).
[e]based upon tissue pallor of carcass and/or low packed cell volume (< 0.35).
[f]moderate to severe lymphocytolysis and involution of lymphoid follicles in bursa of Fabricius, evaluated histologically.

Table D3. Common diagnoses in sick or dead juvenile Franklin's gulls in 1999, 2000, and 2001.

Diagnosis	Number of cases											
	1999			2000			2001					
							Transects			Fenced chicks		
	≤ 7 d	> 7 d	Total	≤ 7 d	> 7 d	Total	≤ 7 d	> 7 d	Total	≤ 7 d	> 7 d	Total
	n = 7	*n* = 46	*n* = 53	*n* = 1	*n* = 30	*n* = 31	*n* = 5	*n* = 25	*n* = 30	*n* = 33	*n* = 13	*n* = 46
Staphylococcus aureus septicemia[a]	0/5	24/39	24/44 (54.5%)	0	1/15	1/16 (6.3%)	0	1	1 (3.3%)	0	1	1 (2.2%)
Salmonellosis	0/5	3/39	3/44 (6.8%)	0	0	0	0	0	0	0	0	0
Septicemia/bacteremia other organisms[b] (*Staphylococcus* spp, *S. aureus*-like sp., *Streptococcus* spp., *Pasteurella* spp., *Escherichia coli*)	2/5	5/39	7/44 (15.9%)	0	9/15	9/16 (56.3%)	3	14	17 (56.7%)	12	9	21 (45.7%)
Dermatitis of head[b]	3/6	24/45	27/51 (52.9%)	0	8	8 (25.8%)	1	6	7 (23.3%)	1	3	4 (8.7%)
Pododermatitis[b]	0	24	24 (45.3%)	0	7	7 (22.6%)	0	3	3 (10.0%)	0	2	2 (4.3%)
Schistosome larvae in foot web	0	4/33	4/40 (10.0%)	0	0	0	0	0	0	0	0	0
Pneumonia	3	17/33	20/40 (50.0%)	1	4	5 (16.1%)	1	3	4 (13.3%)	4	4	8 (17.4%)
Inflammation involving the heart	1	13/33	14/40 (35.0%)	0	13	13 (41.9%)	2	5	7 (23.3%)	4	6	10 (21.7%)
Anemia[c]	1	15	16/53 (30.2%)	0	7	7 (22.6%)	1	9	10 (33.3%)	11	3	14 (30.4%)
Coccidiosis[d]	0	15/33	15/40 (37.5%)	0	6	6 (19.4%)	0	1	1 (3.3%)	0	1	1 (2.2%)
Suspected botulism[e]	0	11	11/53 (20.8%)	0	5	5 (16.1%)	0	9	9 (30.0%)	0	0	0
Adenovirus	0	2/33	2/40 (5.0%)	0	1	1 (3.2%)	0	1	1 (3.3%)	0	2	2 (4.3%)

[a]based on gross and histopathological lesions and bacterial culture. Common lesions observed with *S. aureus* septicemia included pneumonia, myocarditis, and pericarditis.
[b]moderate to severe cases only. All cases that were cultured in 1999 grew *S. aureus* in large numbers, usually in pure culture. Of the 24 pododermatitis cases in 1999, seven (29.2%) also had severe osteoarthritis of the proximal limb.
[c]based on tissue pallor of carcass and/or packed cell volume (<0.35).
[d]moderate to marked cases of large intestinal or renal coccidiosis, involving colitis/ureteritis.
[e]all suspected cases of botulism occurred after the first cases of botulism in waterfowl (Chapter 3), with the exception of one gull in 1999, found on June 24, nearly two weeks before the first detected case in waterfowl. This case, however, was not confirmed (i.e., not tested) using the mouse bioassay due to insufficient volume of plasma collected. Of the serum samples from sick gulls found on transects and islands in mid to late July, 8/16, 3/5, and 4/8 tested positive for botulinum toxin in 1999, 2000, and 2001, respectively.

APPENDIX E. ADDITIONAL MODELS DISCUSSED IN CHAPTER 5

Table E1. Alternate or additional models selected for factors affecting mass and CMI responses in Franklin's gull hatchlings tested at 0-2 days of age.

Variable[a]	*b*	SE	χ^2	*P*	*n*
Model E1 – Neonatal mass					226
Constant	0.406	0.142			
Tx (ref = con)	-0.207	0.135	2.352	0.125	
HO2 (ref = HO1)	-0.352	0.142	6.113	0.013	
HO3 (ref = HO1)	-0.562	0.155	13.186	0.0003	
Egg mass	0.297	0.060	24.737	<0.0001	
Model E2 – Neonatal mass					226
Constant	0.403	0.140			
Tx (ref = con)	-0.216	0.133	2.638	0.104	
HO2 (ref = HO1)	-0.318	0.141	5.101	0.024	
HO3 (ref = HO1)	-0.613	0.153	15.967	0.0001	
Total clutch mass	0.315	0.115	7.540	0.006	
Egg mass	0.026	0.115	0.051	0.821	
Model E3 – Neonatal mass					226
Constant	0.412	0.125			
HO2 (ref = HO1)	-0.444	0.163	7.452	0.006	
HO3 (ref = HO1)	-0.611	0.175	12.228	0.0005	
LO2 (ref = LO1)	-0.155	0.155	1.005	0.316	
LO3 (ref = LO1)	-0.148	0.151	0.960	0.327	
Model E4 – Neonatal CMI					225
Constant	-0.255	0.162			
Tx (ref = con)	0.021	0.160	0.018	0.893	
HO2 (ref = HO1)	0.258	0.158	2.673	0.102	
HO3 (ref = HO1)	0.565	0.172	10.793	0.001	
Model E5 – Neonatal CMI					197
Constant	-0.325	0.163			
Tx (ref = con)	0.104	0.158	0.428	0.513	
HO2 (ref = HO1)	0.247	0.163	2.292	0.130	
HO3 (ref = HO1)	0.511	0.183	7.833	0.005	
Neonatal mass	-0.091	0.069	1.725	0.189	
Neonatal CORT for all except HO3·LO3	-0.043	0.071	0.362	0.547	
Neonatal CORT×HO3·LO3 (ref = all except HO3·LO3)	0.367	0.188	3.815	0.051	

a. ref = reference category for categorical variable; con = control group; tx = treatment group; HO = hatching order; LO = laying order; CMI = cell-mediated immune response as measured by PHA (phytohaemagglutinin) skin test; CORT = corticosterone

Table E2. Alternate or additional models selected for factors affecting corticosterone level and CMI response at 2 weeks in prefledgling Franklin's gulls.

Variable[a]	*b*	SE	χ^2	*P*	*n*
Model E6 – CORT at 2 weeks					158
Constant	-0.383	0.179			
Tx (ref = con)	0.123	0.176	0.493	0.483	
HO2 (ref = HO1)	0.356	0.173	4.224	0.040	
HO3 (ref = HO1)	0.512	0.222	5.308	0.021	
BS1 (ref = BS3)	0.608	0.497	1.497	0.221	
BS2 (ref = BS3)	0.356	0.187	3.610	0.057	
Neonatal CORT	0.186	0.079	5.521	0.019	
Model E7 – CORT at 2 weeks					158
Constant	-0.335	0.171			
Tx (ref = con)	0.151	0.166	0.824	0.364	
BS1 (ref = BS3)	0.629	0.483	1.696	0.193	
BS2 (ref = BS3)	0.365	0.181	4.069	0.044	
Neonatal CORT	0.154	0.077	3.977	0.046	
Mass at 2 weeks	-0.249	0.078	10.079	0.002	
HO2 (ref = HO1)	0.266	0.168	2.496	0.114	
HO3 (ref = HO1)	0.323	0.222	2.124	0.145	
Model E8 – CMI at 2 weeks					181
Constant	-0.244	0.184			
Tx (ref = con)	0.345	0.167	4.273	0.039	
HO2 (ref = HO1)	-0.184	0.171	1.159	0.282	
HO3 (ref = HO1)	-0.218	0.236	0.851	0.356	
CID	-0.272	0.091	8.942	0.003	
Mass 2 weeks×HO3	0.268	0.131	4.156	0.042	
Mass 2 weeks×HO1 (ref = mass 2 weeks×HO3)	-0.228	0.189	1.449	0.229	
Mass 2 weeks×HO2 (ref = mass 2 week ×HO3)	-0.325	0.180	3.261	0.071	

a. ref = reference category for categorical variable; con = control group; tx = treatment group; HO = hatching order; BS = brood size; CORT = corticosterone; CMI = cell-mediated immune response as measured by PHA (phytohaemagglutinin) skin test; CID = clutch initiation date

Table E3. Alternate or additional models selected for factors affecting fate of Franklin's gulls within the first week of life, and from 7 days to fledging.

Variable[a]	*b*	SE	OR	OR 95% CL	*z*	*P*	*n*
Model E9 – Fate to 7 days (mortality = 1, survival = 0)							208
Constant	-6.842	1.947					
Tx (ref = con)	1.891	1.035	6.6	0.87 - 50.4	1.83	0.068	
HO2 (ref = HO1)	0.839	1.284	2.3	0.19 - 28.7	0.65	0.514	
HO3 (ref = HO1)	3.493	1.283	32.9	2.7 - 406.5	2.72	0.006	
Neonatal mass	-1.822	0.896	0.16	0.03 - 0.94	-2.03	0.042	
Neonatal CMI	0.481	0.334	1.6	0.84 - 3.1	1.44	0.149	
Model E10 – Fate to 7 days (mortality = 1, survival = 0)							190
Constant	-6.462	2.023					
Tx (ref = con)	1.897	1.090	6.7	0.79 - 56.5	1.74	0.082	
HO2 (ref = HO1)	0.482	1.137	1.6	0.17 - 15.0	0.42	0.672	
HO3 (ref = HO1)	3.082	1.175	21.8	2.2 - 218.1	2.62	0.009	
Neonatal mass	-1.905	0.936	0.15	0.02 - 0.93	-2.04	0.042	
Neonatal CORT	0.473	0.337	1.6	0.83 - 3.1	1.40	0.161	
Model E11 – Fate after 7 days (mortality = 1, survival = 0)							175
Constant	-5.878	1.452					
Tx (ref = con)	0.793	0.916	2.2	0.37 - 13.3	0.87	0.387	
HO2 (ref = HO1)	1.067	1.237	2.9	0.26 - 32.8	0.86	0.388	
HO3 (ref = HO1)	2.863	1.304	17.5	1.4 - 225.6	2.20	0.028	
Neonatal mass	-1.132	0.580	0.32	0.10 - 1.0	-1.95	0.051	
IGR	-0.940	0.347	0.39	0.20 - 0.77	-2.71	0.007	
Neonatal CMI	-1.519	0.532	0.22	0.08 - 0.62	-2.86	0.004	

a. ref = reference category for categorical variable; con = control group; tx = treatment group; HO = hatching order; CORT = corticosterone; CMI = cell-mediated immune response as measured by PHA (phytohaemagglutinin) skin test; OR = odds ratio

APPENDIX F. MODELS EVALUATED IN CHAPTER 5

Table F1. Set of candidate models considered to evaluate relative importance of factors affecting mass, CMI responses, and corticosterone level in Franklin's gull hatchlings at 0-2 days of age.

Model[a]	ΔAIC[b]	AIC	*k*	*n*
Neonatal mass				
Group, HO, TCM (Model 1)	0	598	5	226
Group, HO, TCM, egg mass (Model E2)	2	600	6	226
Group, HO, egg volume	4	602	5	226
Group, HO, TCM, HO × group	6	604	10	226
Group, HO, egg mass (Model E1)	7	605	5	226
Group, HO	26	624	4	226
HO, LO (Model E3)	30	628	5	226
LO	39	637	2	226
Null	40	638	1	226
Neonatal CMI				
Group, HO, neonatal mass (Model 2)	0	614	5	222
Group, HO, neonatal mass, HO × group	8	622	10	222
Group, HO (Model E4)	16	630	4	222
Group, HO, neonatal mass, neonatal CORT, neonatal CORT × HO3·LO3 (Model E5)	0	544	7	197
Group, HO, neonatal mass, neonatal CORT	2	546	6	197
Group, HO, neonatal CORT, neonatal CORT×HO3·LO3	16	560	6	197
Null	-	634	1	225
Neonatal CORT				
HO3·LO3 (Model 3)	0	577	2	204
Group, HO3·LO3	2	579	3	204
Group, HO, LO	6	583	5	204
Group, HO, LO, egg mass	6	583	6	204
HO, LO, HO × LO	15	592	13	204
Group, HO, LO, HO × LO	15	592	14	204
Group, HO, LO, HO × group	16	593	11	204
Null	4	581	1	204

a. Group refers to treatment vs. control, HO = hatching order, TCM = total clutch mass, LO = laying order, CMI = cell-mediated immune response as measured by PHA (phytohaemagglutinin) skin test; CORT = corticosterone.

b. ΔAICs are employed to rank models with the same sample size. *k* is the number of parameters included in the model.

Table F2. Set of candidate models considered to evaluate relative importance of factors affecting mass, growth rate, corticosterone level, and CMI responses in 2-week-old Franklin's gulls, and humoral response in 3-week-old Franklin's gulls.

Model[a]	ΔAIC[b]	AIC	*k*	*n*
Mass at 2 weeks				
Group, HO, neonatal CMI, neonatal mass, CORT at 2 wks (Model 4)	0	448	7	174
Group, HO, neonatal CMI, neonatal mass, CORT at 2 wks, HO × neonatal CMI	2	450	9	174
Group, HO, neonatal CMI, neonatal mass, CORT at 2 wks, HO × neonatal mass	3	451	9	174
Group, HO, neonatal CMI, neonatal mass, CORT at 2 wks, HO × CORT at 2 wks	3	451	9	174
Group, HO, neonatal CMI, neonatal mass, CORT at 2 wks, HO × group	8	456	12	174
Null	-	520	1	183
IGR to 2 weeks				
Group, HO, neonatal CMI, CORT at 2 wks, CID (Model 5)	0	449	7	174
Group, HO, neonatal CMI, CORT at 2 wks, HO × CORT at 2 wks	4	453	9	174
Group, HO, neonatal CMI, CORT at 2 wks, CID, HO × neonatal CMI	4	453	9	174
Group, HO, neonatal CMI, CORT at 2 wks, CID, HO × CORT at 2 wks, HO × neonatal CMI	8	457	11	174
Group, HO, neonatal CMI, CORT at 2 wks, CID, HO × group	9	458	12	174
Null	-	508	1	179
CORT at 2 weeks				
Group, BS, neonatal CORT, mass at 2 wks (Model 6)	0	440	6	158
Group, BS, neonatal CORT, mass at 2 wks, HO (Model E7)	1	441	8	158
Group, BS, neonatal CORT, HO (Model E6)	8	448	7	158
Group, BS, neonatal CORT, HO, HO × group	16	456	12	158
Null	-	512	1	180
CMI at 2 weeks				
Group, HO, CID, IGR, HO × IGR (Model 7)	0	494	8	177
Group, HO, CID, IGR, HO × IGR, HO × group	8	502	13	177
Group, HO, CID, mass at 2 wks, HO × mass at 2 wks (Model E8)	0	507	8	181
Group, HO, CID, mass at 2 wks	0	507	6	181
Group, HO, CID, mass at 2 wks, HO × mass at 2 wks, HO × group	8	515	13	181
Null	-	514	1	181
Humoral response at 3 weeks				
Group, LO, CORT at 2 wks, neonatal testosterone, CID, TMT growth 2 to 3 wks (Model 8)	0	383	9	144
Group, LO, CORT at 2 wks, neonatal testosterone, CID, TMT growth 2 to 3 wks, egg vol	0	383	10	144
Group, CORT at 2 wks, neonatal testosterone, CID, TMT growth 2 to 3 wks, egg vol	1	384	8	144
Group, CORT at 2 wks, neonatal testosterone, CID, TMT growth 2 to 3 wks	2	385	7	144
Group, LO, CORT at 2 wks, neonatal testosterone, CID	5	388	8	144
Group, LO, CORT at 2 wks, neonatal testosterone, CID, TMT growth 2 to 3 wks, egg vol, LO × group	7	390	15	144
Group, LO, CORT at 2 wks, neonatal testosterone, CID	0	388	8	145
Group, CORT at 2 wks, neonatal testosterone, CID	2	390	6	145
Null	-	496	1	178

a. Group refers to treatment vs. control, HO = hatching order, CMI = cell-mediated immune response as measured by PHA (phytohaemagglutinin) skin test, CORT = corticosterone, CID = clutch initiation date, BS = brood size, IGR = instantaneous growth rate, LO = laying order, TMT = tarsometatarsus length., vol = volume

b. ΔAICs are employed to rank models with the same sample size. *k* is the number of parameters included in the model.

Table F3. Set of candidate of models explored to evaluate factors affecting fate of Franklin's gulls within the first week of life, and from 7 days old to fledging.

Model[a]	ΔAIC[b]	AIC	*k*	*n*
Fate to 7 days[c]				
Group, HO, neonatal mass (Model 9)	-	58	5	212
Group, HO, neonatal mass, neonatal CORT, neonatal CMI	-	55	7	186
Group, HO, neonatal mass, neonatal CMI (Model E9)	-	56	6	208
Group, HO, neonatal mass, neonatal CORT (Model E10)	-	57	6	190
Group, HO, neonatal CMI	-	59	5	210
Null	-	93	1	229
Fate after 7 days[c]				
Group, HO, neonatal mass, IGR, neonatal CMI (Model 10)	0	38	7	175
Group, HO, IGR, neonatal CMI (Model E11)	1	39	6	175
Group, HO, neonatal mass, mass at 2 wks, neonatal CMI	2	40	7	175
Group, HO, neonatal mass, IGR, CMI at 2 wks	7	45	7	175
Group, HO, neonatal mass, mass at 2 wks, CMI at 2 wks	9	47	7	175
Group, HO, mass at 2 weeks, neonatal CMI	-	39	6	176
Group, HO, neonatal mass, mass at 2 wks	-	46	6	177
Null	-	75	1	198

a. Group refers to treatment vs. control, HO = hatching order, CMI = cell-mediated immune response, CORT = corticosterone, IGR = instantaneous growth rate.

b. ΔAICs are employed to rank models with the same sample size. *k* is the number of parameters included in the model.

c. Note: models examining interaction between group and HO, or HO and LO failed to converge.